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LETHAL TOXINS OF HÆMOLYTIC STREPTOCOCCI AND THEIR ANTIBODIES.

E. W. TODD.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

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THERE are at least three toxins of hæmolytic streptococci which are lethal to animals when injected intravenously. (1) Parish and Okell (1927), Hartley (1928) and Pulvertaft (1928) showed that erythrogenic (Dick) toxin is lethal to rabbits, and that protection is afforded by the appropriate antitoxin. Pulvertaft found that erythrogenic toxin with an *m.l.d.* for rabbits of 2·5 c.c. had no effect on mice when injected intravenously in a dose of 0·5 c.c. Hartley found that his concentrated toxin was lethal to rabbits, mice and young white rats when injected intravenously in a dose of 0·5 c.c. to 0·2 c.c. (2) Weld (1934, 1935) prepared a lethal toxin for mice, which is also a powerful hæmolysin, by extracting living hæmolytic streptococci with normal serum and subsequently filtering the serum. (3) Hæmolytic filtrates from cultures grown in Hewitt's glucose-bicarbonate-broth are lethal to mice when injected intravenously.

In this paper the lethal effects of the hæmolytic toxins and the protective action of their antibodies are described. Todd (1938, 1939) showed that group A hæmolytic streptococci form two varieties of streptolysin which are neutralized *in vitro* by different antibodies. They have been named respectively streptolysin O to indicate sensitivity to oxidation and streptolysin S to indicate extractability in serum. Weld's "hæmotoxin" is a mixture of both forms of streptolysin, and attempts to prepare streptolysin S free from streptolysin O have not been successful. On the other hand, reduced filtrates from cultures grown in Hewitt's glucose-bicarbonate-broth contain streptolysin O free from streptolysin S. Antistreptolysin O is prepared by immunizing animals with streptolysin O. Antistreptolysin S is prepared by immunizing animals with living cultures of hæmolytic streptococci; and the sera prepared in this way contain both antistreptolysin S and antistreptolysin O. The hæmolytic toxins available for protection experiments are therefore streptolysin O and Weld's "hæmotoxin" which contains both forms of streptolysin;

the sera available are antistreptolysin O and sera containing both the antistreptolysins. All the sera used in the following protection tests were prepared from the strain E 14 M.A.. Group A, Type 10.

THE PROTECTION OF MICE AGAINST WELD'S HÆMOTOXIN BY ANTISTREPTOLYSIN S.

Only small numbers of mice could be used in experiments on protection against Weld's hæmotoxin owing to the small quantity of high-titred antistreptolysin S serum available. Nevertheless, the results are sufficiently clear-cut to show that antistreptolysin S does protect mice against Weld's hæmotoxin. In the first experiment 21 mice weighing between 30 and 35 g. were divided into 7 groups of 3 mice. The mice belonging to 4 of these groups received 0·5 c.c. of four different sera intraperitoneally and the remainder did not receive any serum. The sera were (1) normal rabbit serum; (2) low titre antistreptolysin S serum from the same rabbit after immunization with living culture; (3) high titre antistreptolysin S serum; (4) concentrated antistreptolysin O serum from a horse immunized with streptococcal filtrates. All the sera were inactivated at 56° C. for 30 minutes before inoculation. Each mouse received an intravenous test dose of Weld's hæmotoxin, prepared from the strain E 14 M.A. Three groups of normal mice, which received no serum, were given diminishing doses of hæmotoxin to obtain some idea of the minimal lethal dose. Table I gives the result of this experiment.

TABLE I.—*The Protection of Mice against Weld's Hæmotoxin by Antistreptolysin S.*

Times of death of mice after receiving—									
0·5 c.c. of sera and 0·5 c.c. of hæmotoxin.						No serum and following doses of hæmotoxin.			
Serum No. 1.	Serum No. 2.	Serum No. 3.	Serum No. 4.			0·5 c.c.	0·25 c.c.	0·1 c.c.	
5 min.	3 min.	S.	3 min.			10 min.	8 hr.	26 hr.	
5 „	3 „	S.	14 „			3 „	3 min.	28 „	
14 „	9 „	S.	3 „			9 „	4 „	7 min.	

Serum No. 1	contained	<1	unit	A.S.O.	per c.c.	and	4	units	A.S.S. per c.c.
„ 2	„	300	units	„	„		8	„	„ „
„ 3	„	300	„	„	„		400	„	„ „
„ 4	„	50,000	„	„	„		2	„	„ „

S. = survived. A.S.O. = antistreptolysin O. A.S.S. = antistreptolysin S. The surviving mice were kept under observation for three months; they all remained well and gained weight. *m.h.d.* of hæmotoxin = 0·001 c.c., which was completely neutralized *in vitro* by 0·004 c.c. of serum No. 3.

The only survivors in the whole experiment were mice which received high-titred antistreptolysin S serum; high-titred antistreptolysin O serum did not protect against Weld's hæmotoxin. Two of the survivors had no toxic symptoms and were never sick; the third developed hæmoglobinuria less than two hours after the dose of hæmotoxin, but within the next two hours the urine had returned to a normal light yellow colour. This transient hæmoglobinuria is in marked contrast to the prolonged hæmoglobinuria seen in

TABLE II.—*The Effect of Antistreptolysin S on Hæmoglobinuria Caused by Weld's Hæmotoxin.*

Duration of hæmoglobinuria after mice received—												
0·5 c.c. of sera and 0·5 c.c. of hæmotoxin.							No serum and following doses of hæmotoxin.					
Serum No. 1.	Serum No. 2.	Serum No. 3.	Serum No. 4.	Serum No. 5.			0·5 c.c.		0·25 c.c.		0·1 c.c.	
60 hr.*	60 hr.*	10 hr. S.H.	S.	16 hr.*			60 hr.*		10 hr. S.H.		4 hr.	S.H.
60 ,, *	50 ,, *	6 ,, S.H.	S.	17 ,, *			60 ,, *		10 ,, S.H.		7 ,,	S.H.

Serum No. 1 contained <1 unit A.S.O. per c.c. and 5 units A.S.S. per c.c.

,,	2	,,	125 units	,,	,,	40	,,	,,	,,		
,,	3	,,	125	,,	,,	80	,,	,,	,,		
,,	4	,,	300	,,	,,	400	,,	,,	,,		
,,	5	,,	50,000	,,	,,	2	,,	,,	,,		

Times = approximate duration of hæmoglobinuria. H. = hæmoglobinuria. * = mouse died. S. = mouse survived. All the mice which died had continuous hæmoglobinuria up to the time of death.

two of the unprotected mice which, after receiving 0·1 c.c. of hæmotoxin, both passed dark red urine up to the time of death.

A less powerful hæmotoxin which caused prolonged hæmoglobinuria and delayed death was used in the next experiment in order to study the effect of antistreptolysin S on hæmoglobinuria. This hæmotoxin, which had an *m.h.d.* of 0·005 c.c., was prepared from the strain "Gay", Group A, Type 15. Normal rabbit serum, concentrated antistreptolysin O serum and three antistreptolysin S sera containing respectively 40, 80 and 400 units were inactivated at 56° C.; they were then injected intraperitoneally into mice weighing 30 to 40 g. The mice subsequently received 0·5 c.c. of hæmotoxin intravenously. Table II gives the result of this experiment.

The only mice in the whole experiment which remained entirely free from toxic symptoms were those which received high-titred antistreptolysin S serum. This is in agreement with the results of neutralization tests *in vitro*, which showed that 0·032 c.c. of the serum containing 40 units of antistreptolysin S completely neutralized 0·005 c.c. (1 *m.h.d.*) of the hæmotoxin; therefore 3·2 c.c. of this serum would be required for complete neutralization of the test dose of hæmotoxin. The mice actually received 0·5 c.c. of serum, leaving a theoretical balance of unneutralized hæmotoxin of 0·42 c.c., and they did not show any signs of protection.

The dose of the serum containing 80 units of antistreptolysin S which neutralized 1 *m.h.d.* of the hæmotoxin *in vitro* was 0·016 c.c.; therefore 1·6 c.c. of serum would be required to neutralize the test dose of hæmotoxin. The mice which received 0·5 c.c. of this serum, leaving a theoretical balance of hæmotoxin of 0·34 c.c., suffered from transient hæmoglobinuria but recovered and survived.

The dose of serum containing 400 units of antistreptolysin S which neutralized 1 *m.h.d.* of hæmotoxin *in vitro* was 0·0032 c.c.; therefore 0·32 c.c. of serum would be required to neutralize the test dose of hæmotoxin. The mice which received 0·5 c.c. of this serum were therefore given an excess of antistreptolysin S and they showed no symptoms.

The Time Factor in Passive Protection of Mice against Weld's Hæmotoxin.

In order to investigate the time factor in passive protection against hæmotoxin, 9 mice were inoculated intraperitoneally with 0·5 c.c. of inactivated serum containing 400 units of antistreptolysin S per c.c.; they then received, at intervals of about 15 minutes, intravenous doses of 0·5 c.c. of hæmotoxin prepared from the strain E 14 M.A.

With an interval of 3 minutes between serum and hæmotoxin the mouse died in 4 minutes; with an interval of 15 minutes the mouse died in 11 minutes; with intervals of 30, 45, 60 and 75 minutes the mice died in about 60 hours; with intervals of more than 75 minutes the mice survived. Twelve control mice received no serum and 0·5 c.c. of hæmotoxin; their average time of death was 4 minutes, the longest time being 7 minutes and the shortest time 2 minutes. Three control mice received 0·25 c.c. of hæmotoxin; they died in 6 minutes, 17 minutes and about 10 hours. Three control mice received 0·1 c.c. of hæmotoxin; they all died in about 18 hours. The hæmotoxin had

an *m.h.d.* of 0·0016 c.c. This experiment shows that the antistreptolysin S should be given about 2 hours before the test dose of hæmotoxin.

The Relative Proportions of Streptolysin S and Streptolysin O in Weld's Hæmotoxin.

The experiments already quoted on protection of mice against Weld's hæmotoxin show that antistreptolysin O had no protective action, although the hæmotoxin contains both forms of streptolysin (Todd, 1938, 1939). The following experiments were therefore done to determine the relative proportions of streptolysin S and streptolysin O in hæmotoxin prepared from the strain E 14 M.A.

Experiment A.—Duplicate titrations of the minimal hæmolytic dose of the hæmotoxin were set up; one titration had 500 units of antistreptolysin O added to each tube, while the other served as a control. The two titrations gave identical results, showing that the hæmotoxin contained a preponderance of streptolysin S.

Experiment B.—The converse process of neutralizing the streptolysin S in the hæmotoxin and titrating the residual streptolysin O is more difficult, since untreated antistreptolysin S sera always contain antistreptolysin O. In the following experiment 1·0 c.c. of serum containing 200 units of antistreptolysin S and 300 units of antistreptolysin O was mixed with 300 units of streptolysin O in 15·0 c.c. of filtrate. The resulting mixture, which was found to contain 1·6 units per c.c. of antistreptolysin O and 12·5 units per c.c. of antistreptolysin S, was used to determine the relative proportions of streptolysin O and streptolysin S in hæmotoxin prepared from the strain E 14 M.A. It was found that the hæmotoxin contained 300 units of streptolysin S per c.c. and less than 8 units of streptolysin O per c.c. The hæmolytic action of the hæmotoxin was therefore almost entirely due to streptolysin S, and this probably explains why antistreptolysin O failed to give any protection against the lethal action of hæmotoxin in mice.

The Action of Foreign Protein on Mice.

The effect of the large amount of foreign protein which the mice received in the protection tests described above was investigated by a control experiment in which inactivated normal rabbit serum was injected intraperitoneally instead of antistreptolysin S serum, and inactivated normal horse serum was injected intravenously instead of hæmotoxin. Nine mice were treated in this way; they all remained well apart from slight initial attacks of shivering.

THE PROTECTION OF MICE AGAINST STREPTOLYSIN O BY ANTISTREPTOLYSIN O.

Preparations of streptolysin O which have a high hæmolytic titre are lethal to mice when injected intravenously. On the other hand the following substances have no lethal action on mice: (1) Streptolysin O, which has been rendered non-hæmolytic by heating for 30 minutes at 56° C. (2) Non-hæmolytic filtrates prepared from strains of hæmolytic streptococci which do

not form streptolysin O in glucose-bicarbonate-broth. (3) Broth reduced with sodium hydrosulphite in the same way as streptolysin O.

In the following experiments mice were given intraperitoneal doses of antistreptolysin O serum, followed about 16 hours later by intravenous doses of streptolysin O which had been reduced with 0·1 per cent. sodium hydrosulphite and stored at 2° C. under vaseline seal for about 3 months. A tube of the lysin was taken from the refrigerator; the vaseline seal was removed; the liquid was decanted and allowed to stand at room temperature for 20 minutes. Intravenous injections were then continued for about 1 hour, a fresh tube of lysin being opened for any subsequent inoculations. These precautions are necessary, as the lysin is uncertain in its lethal action when freshly opened and after standing for a long period at room temperature. Table III gives the result of an experiment in which streptolysin O prepared from three group A strains was used to test the protective action of antistreptolysin O serum prepared from the strain E 14 M.A., Group A, Type 10. All the mice weighed between 25 and 30 g.

TABLE III.—*The Protection of Mice Against Streptolysin O by Antistreptolysin O.*

Times of death of mice after receiving—					
0·5 c.c. of serum and 0·5 c.c. of reduced filtrate.			No serum and 0·5 c.c. of reduced filtrate.		
R.F. No. 1.	R.F. No. 2.	R.F. No. 3.	R.F. No. 1.	R.F. No. 2.	R.F. No. 3.	R.F. No. 4.	R.F. No. 5.
S.	S.	S.	1 min.	17 min.	S.H.	S.	S.
S.	S.	S.	4 ,,	5 ,,	4 hr.	S.	S.
S.	S.	S.	3 ,,	16 ,,	7 min.	S.	S.
S.	S.	S.	2 ,,	5 ,,	2 ,,	S.	S.
S.H.	S.	S.	2 ,,	5 ,,	2 ,,	S.	S.
S.	S.H.	S.	5 ,,	1 ,,	S.H.	S.	S.

R.F. No. 1 = reduced filtrate from strain "Richards" Type 3. *m.h.d.* = 0·02 c.c.
,, 2 = ,, ,, ,, ,, "Lewis" ,, 3. ,, = 0·02 ,,
,, 3 = ,, ,, ,, ,, 089 Untyped. ,, = 0·03 ,,
,, 4 = ,, ,, ,, ,, R 504 Type 3. ,, = 0·06 ,,
,, 5 = ,, ,, ,, ,, S.F. 51 ,, 3. ,, > 0·5 ,,

Serum contained 50,000 antistreptolysin O units per c.c. S. = mice survived.
H. = hæmoglobinuria.

The serum protected mice against each of the three preparations of streptolysin O. Two of the protected mice had transient hæmoglobinuria, which continued for 2 or 3 hours, while the remainder showed no toxic symptoms. Five reduced filtrates, prepared from different strains, were tested on unprotected mice; their lethal effects were roughly proportional to their hæmolytic titres. Filtrates Nos. 1 and 2 killed all the mice; No. 3 killed 4 mice, and the 2 survivors developed hæmoglobinuria which continued for more than 7 hours; No. 4, which had a moderately high hæmolytic titre, did not cause hæmoglobinuria in any of the mice, although in a subsequent experiment (see Table IV) 0·1 c.c. of reduced filtrate with an *m.h.d.* of 0·02 c.c. caused hæmoglobinuria in all the mice; No. 5, which was non-hæmolytic, also caused no symptoms.

TABLE IV.—*Titration of Protective Power of Various Sera against Streptolysin O.*

Dilutions of sera.	Times of death of mice after receiving— 0·5 c.c. of serum dilutions + 0·5 c.c. of streptolysin O. Serum No. 1.	Serum No. 2.	Serum No. 3.	Serum No. 4.	Serum No. 5.	Serum No. 6.	Serum No. 7.	Serum No. 8.	Serum No. 9.	No serum + following doses of streptolysin O. 0·5 c.c.	0·25 c.c.	0·1 c.c.
1 in 1	S.	S.	S.	S.	17 hr.	S.H.	50 min.	5 min.	1 min.	3 min.	S.H.	S.H.
1 „ 10	S.	S.	S.H.	S.H.	2 min.	2 min.	1 „	1 „	1 „	1 „	S.H.	S.H.
1 „ 100	S.	S.H.	48 min.	2 min.	1 „	1 „	2 „	2 „	4 „	1 „	3 min.	S.H.
1 „ 1000	29 min.	1 min.	2 „	5 „	1 „	1 „	1 „	1 „	1 „	1 „	7 „	S.H.
1 „ 10,000	1 „	1 „	9 „	4 „	1 „	2 „	8 „	2 „	7 „	1 „	8 „	S.H.

Horse-serum	No. 1	contained	50,000	units	A.S.O.	per c.c.;	2 units A.S.S. per c.c.; 300 units A.T. per c.c.
„	„ 2	„	20,000	„	„	„	10 „ „ „ 20 „ „ „
„	„ 3	„	8,300	„	„	„	3 „ „ „ 400 „ „ „
„	„ 4	„	5,000	„	„	„	4 „ „ „ 600 „ „ „
„	„ 5	„	160	„	„	„	3 „ „ „
Rabbit	„ 6	„	400	„	„	„	4 „ „ „
„	„ 7	„	250	„	„	„	300 „ „ „
„	„ 8	„	200	„	„	„	200 „ „ „
„	„ 9	„	<1 unit	„	„	„	4 „ „ „

S. = mice survived. H. = hæmoglobinuria in surviving mice. Other survivors had no hæmoglobinuria. A.S.O. = antistreptolysin O. A.S.S. = antistreptolysin S. A.T. = Washington units of scarlet fever antitoxin.

In the next experiment the protective power of diminishing doses of various sera was tested against streptolysin O with an *m.h.d.* of 0·02 c.c. prepared from the strain " Richards ". Nine sera were used in this protection test. Sera Nos. 1 to 4 were concentrated sera from horses immunized with streptococcal filtrates. Serum No. 5 was concentrated serum from horses immunized with diphtheria toxoid. Sera Nos. 6 to 8 were unconcentrated sera from rabbits immunized with living cultures of hæmolytic streptococci. Serum No. 9 was normal rabbit serum. The results of the protection test are given in Table IV.

The protective power of each serum was proportional to its antistreptolysin O titre, although concentrated horse-serum was slightly more efficient than unconcentrated rabbit serum. Mice which received 100 units or more of antistreptolysin O in concentrated horse-serum survived, while those which received 80 units or less did not survive. On the other hand the mouse which received 200 units of antistreptolysin O in rabbit serum survived, while those which received 125 units or less did not survive. The hæmolytic filtrate used in the protection test was found by titration against standard antistreptolysin O serum *in vitro* to contain 20 units of streptolysin O per c.c. ; it did not contain any active streptolysin S. It was therefore necessary to give mice 100 units of antistreptolysin O to protect them against 10 units of streptolysin O, with an interval of 16 hours between serum and lysin. No better protection was obtained when the serum was given 2 hours before the test dose of streptolysin O.

There is no evidence that either antistreptolysin S or antibody to erythrogenic toxin have any protective action against streptolysin O.

PROTECTION EXPERIMENTS AGAINST LIVING CULTURES OF HÆMOLYTIC STREPTOCOCCI.

The Effect of Antistreptolysin O on Infection of Mice by Hæmolytic Streptococci.

It has been shown (Todd, 1935) that high titre antistreptolysin O sera protect mice to a limited extent against infection by either homologous or heterologous types of group A hæmolytic streptococci. To obtain protection the serum must be given about 16 hours before the test dose of culture. This protective action remains unexplained, and although it does not appear to be definitely correlated with the antistreptolysin O titre of the serum, yet it is not dependent on any of the following antibodies—antistreptolysin S, antifibrinolysin, antitoxin (erythrogenic), flocculating antibody, type-specific bacterial antibody (anti-M), group-specific bacterial antibody (anti-C), or antibody to " nucleo-protein " (anti-P).

The Effect of Antistreptolysin S on Infection of Mice by Hæmolytic Streptococci.

In the following experiment on the protection of mice by antistreptolysin S against living hæmolytic streptococci the serum was injected subcutaneously, and within an hour the test dose of living culture, diluted in Ringer's solution, was inoculated intraperitoneally. In order to avoid type-specific antibacterial protection the sera were prepared with a type 10 strain (E 14 M.A.),

while the test organism was a type 3 strain (Richards). Four sera were used in this protection test. Serum No. 1 was normal rabbit serum. Sera Nos. 2 and 3 were from rabbits immunized with living hæmolytic streptococci. Serum No. 4 was concentrated serum from horses immunized with streptococcal filtrates. Table V shows that there was no protection by any of the sera.

TABLE V.—*Protection Experiment with Antistreptolysin S Serum Against Living Hæmolytic Streptococci. Strain Richards.*

Times of death of mice after receiving—						
0·5 c.c. of serum + 10^{-5} c.c. of culture.				No serum + following doses of culture.		
Serum No. 1.	Serum No. 2.	Serum No. 3.	Serum No. 4.	10^{-5} c.c.	10^{-6} c.c.	10^{-7} c.c.
1 day	1 day	1 day	1 day	1 day	1 day	S
1 ,,	2 days	1 ,,	2 days	2 days	2 days	S.
1 ,,	2 ,,	2 days	2 ,,	2 ,,	2 ,,	S.
2 days	2 ,,	2 ,,	2 ,,	2 ,,	S.	S.
2 ,,	2 ,,	2 ,,	2 ,,	1 day	S.	S.
3 ,,	2 ,,	2 ,,	3 ,,	S.	S.	S.

Serum No. 1 contained <1 unit A.S.O. per c.c.; and 5 units A.S.S. per c.c.
,, 2 ,, 300 units ,, ,, 8 ,, ,, ,,
,, 3 ,, 300 ,, ,, ,, 400 ,, ,, ,,
,, 4 ,, 50,000 ,, ,, ,, 2 ,, ,, ,,

10^{-6} c.c. of culture developed about 60 colonies when plated in blood agar.
Pure cultures of hæmolytic streptococci were obtained from the hearts' blood of all the dead mice.

A second protection test was done in which the sera were inoculated intraperitoneally 18 hours before the test doses of culture. The experiment was otherwise the same as that shown in Table V. There was again no evidence of protection against virulent streptococci by antistreptolysin S, although concentrated antistreptolysin O serum protected the mice under these conditions as previously described.

The Combined Effect of Antistreptolysin O and Antistreptolysin S on Infection of Mice by Hæmolytic Streptococci.

The following experiment was done to determine whether antistreptolysin O and antistreptolysin S acting together give more effective protection than antistreptolysin O acting alone. Mice were inoculated intraperitoneally with 0·25 c.c. of the following sera: No. 1 concentrated serum from horses immunized with diphtheria toxoid; No. 2 concentrated serum from horses immunized with streptolysin O; No. 3 normal rabbit serum. Mice were also inoculated with 0·5 c.c. of a mixture containing equal parts of high-titred antistreptolysin O and antistreptolysin S sera. Sixteen hours later the mice received, intraperitoneally, 10^{-4} c.c. of a culture of the strain "Richards" diluted in Ringer's solution. Serum No. 4 was unconcentrated serum from a rabbit immunized with living cultures of the strain E 14 M.A. Table VI shows that although antistreptolysin O probably afforded a limited degree of protection, yet this protective action was not enhanced by the addition of antistreptolysin S.

TABLE VI.—*Protection Experiment with Antistreptolysin Sera Against Living Hæmolytic Streptococci. Strain Richards.*

Times of death of mice after receiving—						
0·25 c.c. of serum + 10^{-4} c.c. of culture.					No serum + following doses of culture.	
Serum No. 1 alone.	Serum No. 2 alone.	Serum No. 3 alone.	Serum No. 2 + Serum No. 4.	Serum No. 1 + Serum No. 3.	10^{-4} c.c.	10^{-5} c.c.
1 day	2 days	1 day	2 days	1 day	1 day	S.
2 days	3 ,,	2 days	3 ,,	1 ,,	2 days	S.
2 ,,	3 ,,	2 ,,	3 ,,	2 days	2 ,,	S.
2 ,,	S.	2 ,,	4 ,,	2 ,,	2 ,,	S.
3 ,,	S.	2 ,,	4 ,,	2 ,,	2 ,,	S.
S.	S.	S.	S.	2 ,,	S.	S.

Serum No. 1 contained 160 units A.S.O. per c.c. and 3 units A.S.S. per c.c.
,, 2 ,, 50,000 ,, ,, ,, 2 ,, ,, ,,
,, 3 ,, <1 unit ,, ,, 4 ,, ,, ,,
,, 4 ,, 250 units ,, ,, 300 ,, ,, ,,

10^{-6} c.c. of culture developed about 50 colonies when plated in blood agar.
Pure cultures of hæmolytic streptococci were obtained from the hearts' blood of all the dead mice.

DISCUSSION.

The lethal effects of both the streptolysins appear to be proportional to their hæmolytic titres, but the number of *m.h.d.'s* required to cause rapid death of mice is much greater with streptolysin S than with streptolysin O. Weld's hæmotoxin prepared from the strain E 14 M.A. was found to be almost entirely dependent on streptolysin S for its hæmolytic action, and, if 0·5 c.c. was used as the test dose for mice, rapid death occurred when the *m.h.d.* was 0·001 c.c.; when the *m.h.d.* was 0·005 c.c. death was delayed in the majority of mice; and when the *m.h.d.* was 0·02 c.c. hæmoglobinuria was followed by recovery. On the other hand preparations of streptolysin O caused rapid death of mice when the *m.h.d.* was 0·02 c.c. This difference in dosage suggests that the lethal actions of the two streptolysins are not both dependent on intravascular hæmolysis. Weld (1934, 1935), Schlüter and Schmidt (1936) and Hare (1937) have described the post-mortem appearances of animals killed by intravenous doses of Weld's hæmotoxin. The most characteristic post-mortem finding is complete lysis of the animal's red blood-corpuscles. On the other hand intravascular hæmolysis is not conspicuous *post mortem* in mice killed by streptolysin O, although the occurrence of hæmoglobinuria indicates that hæmolysis *in vivo* does occur. Assuming that a mouse's red blood-corpuscles are destroyed by the streptolysins *in vivo* to the same extent as rabbit's blood-cells *in vitro*, then 0·5 c.c. of streptolysin S with an *m.h.d.* of 0·001 c.c. would lyse about 175 billion red cells. As each mouse has about 20 billion red cells, this dose of streptolysin S contains about 8 times more lysin than the quantity required to lyse all the red cells in the animal's body and the post-mortem appearances suggest that complete hæmolysis does actually occur. On the other hand, 0·5 c.c. of streptolysin O with an *m.h.d.* of 0·02 c.c. would lyse only 9 billion red cells, leaving more than half the red corpuscles intact. This is a condition which would probably not cause death,

since the remaining red cells would suffice for tissue respiration, and Weld has shown that lysed red cells inoculated intravenously are not lethal. Additional evidence that mice would survive this degree of hæmolysis is supplied by the fact that 0·5 c.c. of streptolysin S with an *m.h.d.* of 0·02 c.c. causes hæmoglobinuria followed by recovery. It therefore seems probable that the lethal action of streptolysin S is due to intravascular hæmolysis, and that streptolysin O has a different lethal action.

Weld (1934, 1935) and Hare (1937), using hæmotoxins prepared from a number of different strains, were unable to correlate lethal effects with hæmolytic titres. There is very considerable variation in the relative proportions of streptolysin O and streptolysin S produced by different strains of hæmolytic streptococci, and since Weld's hæmotoxin contains both streptolysins, the relation of hæmolytic titre to lethal action will depend on the relative proportions of the two streptolysins. Preparations of hæmotoxin with a high streptolysin O content and low streptolysin S content would be highly lethal with a relatively low hæmolytic titre. Conversely a high streptolysin S content and low streptolysin O content would cause the hæmotoxin to be less lethal in relation to hæmolytic titre. It is probable that the failure of Weld and of Hare to correlate the lethal and hæmolytic effects of their hæmotoxins was due to variations in the proportions of the two streptolysins in different preparations of hæmotoxin.

The lethal action of streptolysin S can be fully explained by intravascular hæmolysis, while the lethal action of streptolysin O appears to be dependent on other factors. When mice, which have been inoculated intravenously with a fatal dose of streptolysin O, remain alive for 5 or 10 minutes, a blood-stained frothy liquid exudes from the nostrils and extensive damage to the lungs is found *post mortem*. This is probably one of the factors which cause death when streptolysin O is injected intravenously. Nevertheless a dose of streptolysin O which is fatal to mice when injected intravenously produces no symptoms when injected subcutaneously or intraperitoneally.

The antibodies of the two streptolysins have been shown by experiments *in vitro* to neutralize the corresponding streptolysins without any cross-neutralization (Todd, 1939). This observation is now confirmed by experiments *in vivo*, which show that antistreptolysin S protects mice against streptolysin S but not against streptolysin O, and that antistreptolysin O protects mice against streptolysin O but not against streptolysin S.

SUMMARY.

1. Group A hæmolytic streptococci produce two distinct varieties of streptolysin which are both lethal to mice :

(*a*) Streptolysin O, which is oxygen-sensitive.

(*b*) Streptolysin S, which is serum-extractable.

These two streptolysins are neutralized by separate antibodies which are entirely unrelated.

2. Antistreptolysin O protects mice against either death or hæmoglobinuria which may be caused by streptolysin O, but it does not protect against streptolysin S.

3. Antistreptolysin S protects mice against either death or hæmoglobinuria which may be caused by streptolysin S, but it does not protect against streptolysin O.

4. Antistreptolysin S does not protect mice against living cultures of hæmolytic streptococci, and it does not enhance the protective action of antistreptolysin O.

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STUDIES ON ANTIRABIC IMMUNIZATION WITH FORMOLIZED CULTURE VIRUS.

I. J. KLIGLER AND H. BERNKOPF.

From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem.

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THE successful cultivation of rabies virus in tissue cultures (Kanazawa, 1936, 1937 ; Webster and Clow, 1936, 1937 ; Bernkopf and Kligler, 1937) has raised the possibility of simplifying antirabic immunization by use of such cultures. Hodes, Lavin and Webster, 1937, have shown that cultures inactivated by suitable exposure to ultra-violet light retain their antigenic capacity and can immunize mice against ten intracerebral doses of test virus.

The experiments reported below were designed to test the immunizing power of cultures treated with formalin. Previous experiments with Rickettsia (Kligler and Aschner, 1934) and with vaccinia virus (Kligler and Bernkopf, 1937) have demonstrated that formalin has the property of inactivating viruses without affecting their antigenic property. It seemed, desirable, therefore, to employ this technique with rabies virus.

EXPERIMENTAL.

Materials and Methods Used.

The cultures used in these experiments were grown according to the procedure described by Webster and Clow. After 4 to 5 days' incubation, the cultures were centrifugalized in large tubes, the supernatent fluid removed,

and the sediment triturated with a glass pestle. The ground material was resuspended in the supernatant fluid and 0·1 per cent. formalin added. As a rule, the titre of the culture was tested by mouse inoculation before adding the formalin; the cultures used contained from 5000 to 50,000 lethal mouse units per c.c. All vaccines were tested for sterility, and also for inactivity by intracerebral inoculation into at least 2 mice.

The experiments may be divided into three groups: One set of experiments was designed to ascertain the immunizing effect of varying doses and number of injections of formolized cultures. In a second series we tested the immunizing effect of a preliminary treatment with inactivated culture virus followed by an intraperitoneal or subcutaneous infection with active brain virus. In the last set we tested the immunizing effect of live culture virus.

All experiments were carried out with mice and the resulting immunity was tested by intracerebral injections of a uniform suspension of mouse-passage virus, prepared by freezing and thawing followed by mild centrifugation. Further details of the procedure are given in connection with the individual experiments.

Immunization Experiments with Formalin Inactivated Culture Virus.

In a preliminary experiment two sets of mice were each given six injections of 0·25 c.c. of culture vaccine at intervals of 2 to 3 days. Thirteen mice received subcutaneous and 5 mice intraperitoneal injections. Twelve days after the last injection the mice were given intracerebral injections of 1 : 100 and 1 : 500 dilutions of test virus. All mice immunized by the subcutaneous route succumbed to a typical infection. Of the mice treated by the intraperitoneal route, two died the next day from injury caused by the intracerebral injection, and of the remaining three, two survived. The titre of the test virus was 1 : 10,000.

It was apparent from this experiment that the subcutaneous route was not suitable for immunization and that the intracerebral doses given constituted too severe a test, since they contained at least 20 to 100 lethal mouse doses.

In the subsequent experiments the inactive virus was injected intraperitoneally. In one series, 9 mice were given 7 injections of 0·5 c.c. of formolized culture (fourth to ninth passage pooled) at intervals of 3 days, while 4 mice received only 1 injection. In a second series 9 mice received 7 injections and 11 mice 5 injections of 0·25 c.c. of the vaccine.

The results are summarized in Table I. Out of a total of 29 mice given 5 to 7 injections of inactivated virus intraperitoneally, 13 (45 per cent.) were fully immune to at least 10 lethal intracerebral mouse units of test virus, and 4 (13·5 per cent.) were partially immune as indicated by a prolonged incubation period (14 to 24 days), 2 or 3 times that of the controls. One group of 5 mice which received only 1 injection of vaccine, succumbed to the test virus in the same period as the controls.

TABLE I.—*Immunization of Mice with Inactive Cultures of Rabies Virus Against an Intracerebral Test Infection.*

Number of experiment.	Number of treatments.	Mode of injection.	Test virus. Interval after vaccination.	Test virus. Test dose.	Number of mice injected.	Results: Number died after days.	Results: Number survived.*
1	7 × 0·5 c.c.	Intraperitoneal	8 days	1 : 100	4	3 (9 days)	1
		Ditto	8 „	1 : 1000	5	3 (10–12 „)	2
	1 × 0·5 „	„	8 „	1 : 1000	5	5 (9–10 „)	0
2	7 × 0·25 „	„	8 „	1 : 1000	9	4 (9–12 „)	3
						2 (14–16 „)	
	5 × 0·25 „	„	8 „	1 : 1000	11	2 (8–9 „)	7
						2 (20–24 „)	
Control.	..	..	..	1 : 1000	10	10 (8–9 „)	0
				1 : 10,000	3	3 (9 „)	0

* Observed at least 4 weeks.

Immunizing Effect of Formalin Inactivated Virus Against an Intraperitoneal Infection with Test Virus.

The intracerebral test of immunity is obviously a very severe criterion of immunity. The subsequent experiments were, therefore, intended to ascertain the immunity against an intraperitoneal or subcutaneous injection of test virus. Preliminary tests, as well as later controls, indicated that 0·25 c.c. of a 1 : 10 emulsion of our mouse-brain virus injected intraperitoneally killed 60 to 70 per cent. of the mice. Of a total of 40 mice so treated, 33 (67·5 per cent.) died within 10 to 14 days with typical paralysis and 13 (32·5 per cent.) survived.

TABLE II.—*Immunization of Mice with Formalin Inactivated Cultures of Rabies Virus Against an Intraperitoneal or Subcutaneous Test Infection.*

Number of experiment.	Number of injections inactivated culture (intraperitoneally).	Number of mice.	Infection with test virus (0·25 c.c. 1 : 10). Interval after treatment.	Mode of infection.	Died.	Survived.
4	3 × 0·25 c.c.	12	8 days	Intraperitoneal	0	12
	5 × 0·25 „	8	8 „	„	0	8
	7 × 0·25 „	12	8 „	„	0	12
Control	..	6	..	..	4	2
5	3 × 0·25 c.c.	4	15 „	Intraperitoneal	0	4
	3 × 1·00 „	4	15 „	„	0	4
	2 × 0·50 „	2	15 „	„	0	2
	2 × 1·00 „	1	15 „	„	0	1
Control	..	6	..	..	3	3
6	1 × ·025 c.c.	5	14 days	Intraperitoneal	0	5
	2 × 0·25 „	5	14 „	„	0	5
	3 × 0·25 „	5	14 „	„	0	5
Control	..	6	..	Subcutaneous	4	2
7	1 × 0·25 c.c.	5	14 days	„	1	4
	2 × 0·25 „	5	14 „	„	1	4
	3 × 0·25 „	5	14 „	„	0	5
	1 × 1·00 „	5	14 „	„	0	5
	1 × 0·50 „	5	14 „	„	0	4
Control	..	6	..	„	5	1

TABLE III.—*Immunity of Mice Surviving an Intraperitoneal or Subcutaneous Infection with Rabies Virus Against an Intracerebral Infection.*

Preliminary treatment with inactivated virus.	Number of mice surviving subcutaneous or intraperitoneal infection.	Time interval after intraperitoneal or subcutaneous infection.	Re-infected intracerebrally with 1 : 1000 dilution of test-brain virus: Died after infection (days).	Survived.	Remarks.
(Intraperitoneal.)					
3 × 0·25 c.c.	12	19 days	4 (11–12 days)	7	..
			1 (18 „)		
5 × 0·25 „	8	19 „	1 (15 „)	7	..
7 × 0·25 „ (1 : 10).	12	19 „	3 (12 „)	..	2 died from injury on day following infection.
			4 (14–16 „)	3	..
Untreated mice surviving from intraperitoneal infection	2	19 „	1 (18 „)	1	..
Normal controls	10	..	10 (8–9 „)	0	2 additional controls were given 1 : 10,000. Died 9–10 days after infection.
3 × 0·25 c.c.	4	20 days	0	4	..
3 × 1·00 „	4	20 „	0	4	..
2 × 0·50 „	2	20 „	1 ? (8 „)	1	Cause of death not ascertained.
2 × 1·00 „	1	20 „	0	1	..
Untreated mice surviving intraperitoneal infection	3	20 „	1 (15 „)	2	..
Normal controls	4	..	4 (9–10 „)	0	2 control mice also received 1 : 10,000 and died in 9–12 days.
1 × 0·25 c.c.	5	21 days	0	4	1 died from the injection. Surviving mice observed 24 days.
2 × 0·25 „	5	21 „	0	5	..
3 × 0·25 „	5	21 „	0	5	..
Surviving intraperitoneal infection	2	21 „	2 (15–17 days)	0	..
Controls	4	..	4 (8–9 „)	0	..
(Subcutaneous.)					
1 × 0·25 c.c.	4	21 days	3 (8–9 „)	0	1 died from injection.
2 × 0·25 „	4	21 „	1 (17–18 „)	3	..
3 × 0·25 „	5	21 „	0	5	..
1 × 1·00 „	5	21 „	3 (7–10 „)	1	..
			1 (14 „)		
1 × 0·50 „	4	21 „	1 (9 „)	1	..
			2 (13–15 „)		
Surviving subcutaneous infection without previous vaccination	1	21 „	0	1	..
Normal controls	4	..	4 (9–10 „)	0	2 additional control mice received 1 : 10,000 dilution and died in 9–12 days.

Of 10 mice given the same dose subcutaneously 1 survived. The constancy of these results warranted the use of these methods as a test of the immunity resulting from treatment with inactivated virus.

Four sets of experiments were carried out. In 3 of them the test infection was given by the intraperitoneal and in 1 by the subcutaneous route. The results are summarized in Table II.

It will be noted that as little as a single intraperitoneal injection of 0·25 c.c. of inactivated virus suffices to protect the mice against a subsequent infection with a dose of virus which kills over 60 per cent. of the untreated animals.

The animals from the foregoing experiments were subsequently given an intracerebral injection of a 1 : 1000 dilution of test virus in order to ascertain whether survival after an intraperitoneal or subcutaneous infection confers solid immunity against an intracerebral infection. The results are summarized in Table III. The data show that of 46 mice which had received one or more injections of inactivated culture virus followed by an intraperitoneal infection, 38, or 84 per cent. were completely immune to 10 lethal mouse doses given intracerebrally ; while only 5 (or 11 per cent.) succumbed to the infection in the normal time period. On the other hand, mice which received inactivated culture virus diluted (1 : 10) or those which received no treatment at all—although they survived the intraperitoneal infection—possessed only a partial immunity. Of 10 mice receiving diluted vaccine followed by an intraperitoneal infection, 3 only were completely immune and survived the intracerebral infection, and 4 were partially immune. Of 8 non-immunized mice which survived the intraperitoneal infection, 4 resisted the intracerebral test and 4 died after 15 to 18 days.

It is also of interest that among the mice surviving the subcutaneous infection after previous intraperitoneal treatment with vaccine, the degree of immunity was less than among those infected intraperitoneally. Of those receiving one injection of formolized culture prior to the infection (1 × 0·25 c.c. or 1 × 0·5 c.c. or 1 × 1·0 c.c.), only 2 out of 13 survived the intracerebral infection ; of those receiving 2 × 0·25 c.c. or 3 × 0·25 c.c. of the formolized vaccine, 8 out of 9 survived and 1 died after a prolonged incubation period of 17 days.

These results correspond with those in the preliminary experiment which indicated that immunization by the subcutaneous route is less effective than by intraperitoneal treatment.

Immunization with Live Culture Virus.

In one experiment we tested the virulence of culture virus for mice and the subsequent immunity of surviving mice against an intracerebral injection of test-brain virus (1 : 1000). Two sets of 6 mice each were injected with a triturated suspension of live virus. The culture was a twenty-sixth passage and the intracerebral titre was 1 : 100. One group of mice received 0·25 c.c., one group 0·5 c.c., and the third group 1·0 c.c. of the culture. All injections were given intraperitoneally. The mice in the first two groups showed no symptoms ; in the last group 1 mouse developed paralysis 8 days

after the injection and died 2 days later from a typical infection—brain passage confirmed death from rabies.

Twenty days after the treatment with culture virus the mice were given an intracerebral injection of a 1 : 1000 dilution of test virus. In the group which received 0·25 c.c. of culture, 4 out of 6 mice survived and 2 died of a typical infection in 8 days. In the second and third group all mice survived.

It appears from these results that the virus culture which had a low virulence retained its immunizing capacity.

SUMMARY.

Experiments are reported which indicate that formolized tissue cultures of rabies virus possess considerable immunizing capacity for mice, if injected intraperitoneally. The immunity to an intracerebral infection is incomplete ; a little less than half of the mice given 5 to 7 injections of the vaccine possessed a solid immunity to 10 lethal intracerebral mouse doses ; about 42 per cent. possessed no immunity.

Immunity to an intraperitoneal or subcutaneous infection is more easily established. Even a single intraperitoneal treatment with the inactivated culture virus is sufficient to protect mice against an intraperitoneal infection with a dose of fresh virus which kills about two-thirds of the control mice. A similar immunity is conferred against a subcutaneous infection except that a larger amount of vaccine is required.

Of special interest, perhaps, are the data showing that immunized mice which survived an intraperitoneal infection possess a greater degree of immunity than do untreated mice which survived such an infection. Primary treatment with inactive virus affects to a marked extent the immunity of these mice to an intracerebral infection. Mice which received diluted vaccine (1 : 10) or fewer doses possessed a lower degree of immunity than the others, although all of them survived an intraperitoneal or subcutaneous infection with fresh brain virus. Only half of the mice which survived an intraperitoneal infection without previous vaccination were immune to an intracerebral infection, whereas 84 per cent. of previously immunized mice proved immune.

A single dose of a rabies virus culture which had a very low virulence for mice, if injected intraperitoneally, conferred on the treated mice a high degree of immunity against a subsequent intracerebral infection with test-brain virus.

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SPONTANEOUS VARIATION IN THE NEUROTROPIC STRAIN OF YELLOW FEVER VIRUS.

G. M. FINDLAY AND F. O. MACCALLUM.

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IN certain genera of animals and plants, such as *Drosophila* and *Œnothera*, which have received extensive study, spontaneous variation or mutation is by no means uncommon. Among animal and plant viruses a large number of variations have now been described, but, in the case of animal viruses at any rate, variation is usually associated with some environmental change (*cf.* Findlay, 1936). In plant viruses, however, spontaneous variation has not infrequently been observed in the absence of any obvious change of environment. Such spontaneous variations are observed in the appearance of yellow tobacco mosaic in association with ordinary or green tobacco mosaic (McKinney, 1927 and 1929, and Jensen, 1933), of yellow wheat mosaic in association with ordinary or green wheat mosaic (McKinney, 1937), and possibly in other virus infections such as mosaic of brambles, which also exists in two forms, yellow and green (Jones, 1937). Price (1934) reported that tobacco plants infected with cucumber mosaic virus developed bright yellow spots from which different, but closely related, yellow mosaic and necrotic type viruses could be isolated. Among animal viruses the only record of spontaneous variation, unassociated with any environmental change, is that reported by Andrewes and Shope (1936), who found that the rabbit fibroma virus, instead of giving rise to a neoplastic reaction, caused active necrosis. Berry (1938), however, obtained a non-lethal "mutant" strain of virus myxomatosum from heat-inactivated myxoma virus and active fibroma virus. The following observations describe an apparently spontaneous variation in the neurotropic strain of yellow fever virus.

The neurotropic strain of yellow fever virus in which variation occurred was originally produced in 1930 by Theiler by repeated passage of the French strain of pantropic yellow fever virus in mouse brains. When the events to be related took place the virus had been continuously passaged in mouse brains over a period of nearly eight years, during the last five and a quarter years in this laboratory, and some 750 consecutive passages in mouse brains had been made. Routine passage has consisted of the intracerebral inoculation of mice with 0·03 c.c. of a 20 per cent. suspension of infected mouse brain in 1 in 10 serum saline. The mice thus inoculated with this presumably "fixed" neurotropic virus develop nervous symptoms on the fourth day after inoculation, and die usually within 24 hours with symptoms of encephalitis.

In January, 1938, in order to obtain a considerable supply of neurotropic yellow fever virus, a rhesus monkey (No. 1) was inoculated intracerebrally

with 1 c.c. of a 20 per cent. suspension of brain from a mouse infected with the neurotropic yellow fever virus. Previously, when rhesus monkeys had received such an intracerebral injection, no symptoms appeared for from 7 to 9 days, when the temperature rose and death occurred in from 10 to 12 days with symptoms of encephalitis. In the present instance, however, the rhesus monkey developed a temperature of 104·2° F. on the third day and died on the fifth day after inoculation, having failed to develop any symptoms suggestive of nervous involvement. At the post-mortem examination the liver was brownish-yellow in colour and hæmorrhagic material was present in the stomach. The clinical symptoms and pathological changes were those characteristic, not of the neurotropic strain, but of the original pantropic yellow fever virus. Nevertheless, serum and liver from this monkey inoculated intracerebrally into mice produced encephalitis, not after an interval of at least 7 to 9 days, as is the case with the true pantropic strain, but in from 4 to 5 days, the interval characteristic of the established neurotropic strain of yellow fever virus. The evidence thus suggested that the neurotropic strain of yellow fever virus had reverted to pantropism for the rhesus monkey while retaining its neurotropism for the mouse. However, although no pantropic virus was being used in the laboratory at the time, it was thought possible that some error had been made in the inoculation of the monkey. A fortnight later therefore after four further passages in mouse brains, 1 c.c of a 20 per cent. suspension of mouse brain infected with the ordinary passage strain of supposedly fixed neurotropic yellow fever virus was again inoculated intracerebrally into a rhesus monkey (No. 2). Again the temperature rose to 105·2° F. on the third day after inoculation, no nervous symptoms appeared and the animal died 7 days after inoculation. The appearances, both naked-eye and microscopic, were consistent with death from infection with the pantropic yellow fever virus. The blood and liver of this monkey were again inoculated intracerebrally into mice and produced symptoms of encephalitis in from 4 to 5 days. A rhesus monkey (No. 3) was also inoculated intracerebrally with blood from monkey 2 and died on the seventh day with no symptoms of encephalitis, but with viscerotropic lesions characteristic of ordinary yellow fever. Liver and blood from monkey 3 inoculated intracerebrally into mice produced encephalitic symptoms on the fourth day after inoculation, death following in 24 hours. On a third occasion after five further passages in mouse brain the supposedly fixed neurotropic strain of yellow fever virus was injected intracerebrally into a rhesus monkey (No. 4). The temperature rose to 105·8° F. 48 hours after inoculation, and the monkey was moribund on the fourth day after injection, having at no time exhibited nervous symptoms. The liver was necrotic, and altered blood was present in the stomach. Serum from this monkey was passed subcutaneously into monkey 5, which died on the sixth day after injection, with the naked-eye and microscopic appearances characteristic of ordinary yellow fever. Blood and liver from monkeys 4 and 5 injected intracerebrally into mice produced encephalitis on the fourth day after inoculation. Cultures made from the blood of the 5 monkeys failed to show any evidence of bacterial contamination, while the virus present was neutralized, as shown by the mouse protection test, by sera known to contain immune bodies against yellow fever. From the behaviour of this

apparently fixed neurotropic virus it appears that it had reverted to its original pathogenicity for rhesus monkeys, while retaining unimpaired its neurotropic action when inoculated intracerebrally into mice.

In order to determine how long this change had occurred, neurotropic virus which had been frozen and dried a year previously in February, 1937, was tested for its action when injected intracerebrally in a monkey (No. 6). After an intracerebral inoculation of 1 c.c. of a 20 per cent. suspension of mouse brain infected with the dried virus, the monkey developed encephalitic symptoms 10 days after inoculation and was killed 2 days later. No lesions were present in the liver or stomach and virus was absent from the blood-stream. Virus, however, was present in the brain and, on intracerebral injection into a rhesus monkey (No. 7), it again gave rise to a typical encephalitis without signs of viscerotropic lesions.

The evidence thus shows that a change occurred in the fixed neurotropic virus between February, 1937, and January, 1938, despite the fact that the virus had been passaged solely in mouse brains, and had undergone more than 670 consecutive passages in mouse brain without showing any tendency to vary. During the first 20 or so passages of a pantropic strain of yellow fever virus in the brains of mice, the capacity to produce viscerotropic lesions in monkeys is still to some extent retained, but gradually decreases with increase in the number of mouse-brain passages. Stéfanopoulo (1932) on one occasion, however, obtained spontaneous viscerotropic lesions in a rhesus monkey following subcutaneous inoculation with a French strain of neurotropic virus that had undergone 161 passages in mouse brains. The results of intracerebral inoculation of mice with this spontaneously reconverted virus are, however, not recorded. Findlay and Clarke (1935), by repeated intrahepatic passage of the neurotropic strain in rhesus monkeys, were successful in reconverting the neurotropic into the pantropic strain with, at the same time, a decrease in the neurotropic pathogenicity of the virus. The virus, before attempts at reconversion were made, had been passaged rather more than 200 times in mouse brains.

The particular interest of the present observations lies in the fact that, without any environmental change, sufficient pantropism had been regained to produce lesions in the liver and stomach of rhesus monkeys. It has been suggested elsewhere (Findlay and Stern, 1935, and Findlay, 1936) that when ordinary yellow fever virus is injected into a susceptible animal, the inoculum either already contains or, as the result of mutation shortly after inoculation, contains particles of two types. When inoculated subcutaneously into a rhesus monkey the pantropic particles proliferate and kill the monkey with lesions in the viscera; when inoculated intracerebrally into mice or intracerebrally into rhesus monkeys which have received an intraperitoneal inoculation of immune serum the neurotropic particles proliferate and kill the animals with encephalitic lesions. As a result of repeated intracerebral injection the number of pantropic particles gradually decreases and, as a rule, such pantropic particles almost entirely vanish. In the present instance, however, something in the nature of a reversion must have occurred, and, while still growing in nervous tissues and killing mice with encephalitis, sufficient pantropic virus particles must have appeared to ensure the death of a monkey with visceral

lesions. This reversibility may be akin to the reverse mutations very occasionally seen in mutant forms in animals and plants, or to the appearance of adaptative enzymes in bacteria (*cf.* Yudkin, 1938). As in the case of bacteria, absolute reversibility in viruses is thus not fully present, but just as among bacteria no case has so far been recorded of a reversion naturally or experimentally produced, from a highly specialized pathogenic or saprophytic bacillus to the primitive autotrophic type, so among viruses no reversion has occurred to an agent capable of multiplying in the absence of living cells.

Apart from the interest which these observations throw on the constitution of the yellow fever virus they have a strictly practical bearing and emphasize the necessity of testing from time to time any attenuated yellow fever virus that may be used for human immunization, in order to ensure that no increase has occurred either in neurotropic or viscerotropic pathogenicity.

CONCLUSIONS.

1. A strain of neurotropic yellow fever virus that had undergone more than 670 consecutive passages in mouse brains was found to have become pantropic for rhesus monkeys, producing liver necrosis and hæmorrhage in the stomach.

2. The strain was still fully neurotropic for mice.

3. The similarity to a reverse mutation or adaptative enzyme is discussed, and the necessity of testing the pathogenicity from time to time of all strains of yellow fever virus used for human immunization is emphasized.

Intracerebral Injection of Rhesus Monkeys with a Neurotropic Strain of Yellow Fever Virus that had undergone Spontaneous "Reverse Mutation".

No. of monkey.	Inoculum.	Passage No. of virus in mouse brains.	Number of days to death.	Lesions at death.	
				Cerebral.	Hepatic.
1	1 c.c. 20 per cent. suspension mouse brain	753	5	—	+++
2	1 c.c. 20 per cent. suspension mouse brain	757	7	—	+++
3	1 c.c. blood serum monkey 2	—	7	—	+++
4	1 c.c. 20 per cent. suspension mouse brain	762	4	±	+++
5	1 c.c. blood serum monkey 4	—	6	—	+++
6	1 c.c. 20 per cent. suspension mouse brain	671	12	+++	—
7	1 c.c. 20 per cent. suspension brain monkey 6	—	13	+++	—

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THE SPECIFICITY OF ACTIVE IMMUNITY IN MICE AGAINST INFLUENZA VIRUS.

F. M. BURNET.*

From the Walter and Eliza Hall Institute, Melbourne.

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IN previous papers it has been shown that the egg-adapted influenza strain "Melbourne" (M.E.) is normally non-pathogenic for mice, and after intranasal instillation immunizes them solidly against a mouse-pathogenic race of the same strain (M.M.) (Burnet, 1937*a*). A brief report was also made that such immune mice showed a much lower grade of resistance against antigenically different types of human influenza virus (Burnet, 1937*b*). The present paper is concerned with a more detailed study of the specificity of the active immunity induced by administration of living M.E. virus to mice. It is usually considered that active immunity against influenza viruses is much less specific than are the results of neutralization tests with immune sera. Shope (1935), for instance, found that mice immunized (by intraperitoneal injection) against "P.R. 8" were immune to "Swine" influenza virus, which is sharply differentiable from "P.R. 8" by serological tests. Our results, however, indicate that the active immunity produced by infection with M.E. is far more effective against the homologous M.M. and the closely similar "Philadelphia" strain than against serologically dissimilar strains like "W.S."

* Work carried out under grants for research on virus diseases from the Rockefeller Foundation and from the National Health and Medical Research Council.

METHODS.

Mice were immunized by a single administration of 0·05 c.c. of M.E. virus either undiluted (1 egg membrane ground with 5 c.c. broth and spun in an angle centrifuge for 10 minutes), or diluted as far as required in broth. In the earlier experiments, when the M.E. virus was almost completely non-virulent for mice, undiluted virus was used. In the more recent ones, when the virus has shown the atypical increased virulence recently described (Burnet and Lush, 1938) virus diluted 1 : 200 has generally been used. Immunity develops about the eighth day, and the tests have been made as a rule 2 to 3 weeks after the immunizing dose. The test virus strains are maintained by mouse passage. In most experiments the inoculum was prepared from consolidated lungs of mice dead or moribund 4 to 5 days after inoculation, but in the more recent experiments we have found 2-day lungs to provide higher titre virus, which is almost invariably completely free from bacteria.

TABLE I.—*Resistance to Virulent Strains of Influenza Virus of Mice Immunized with Large and Small Doses of "Melbourne" Egg Virus in its Active Phase.*

Test strain.	Dilutions of test virus.					
	Undiluted.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
"Melbourne"						
Normal	..	..	..	$4^5, 4^6, 4^7$	$4^7, 3, 2$	2, 2, 1
Immune (1)	2, 1, 0	0, 0, 0	..	..	..	..
Immune (1 : 100)	1, 0, 0	0, 0, 0	..	..	..	..
"W.S."						
Normal	..	..	..	$4^5, 4^5, 4^7$	$4^6, 4^7$, X	$4^6, 3, 3$
Immune (1)	..	$4^7, 3, 2$	3, 2, 0	2, 2, 1	..	..
Immune (1 : 100)	4^4	$4^5, 4^5, 4^5$	$4^5, 4^6, 4^7$	$4^3, 4^5, 3$	..	..

In both tables lesions in mouse lungs are stated according to the convention used in previous papers. 0 = No lesions. 1, 2, 3 = Increasingly large areas of consolidation found when mouse was killed seven days after inoculation. 4 = Death with complete consolidation on the day shown by the index figure. X = Death from non-specific cause.

TABLE II.—*Immunity of Mice Immunized with "W.S." by Intranasal Route.*

Test strains.	Dilutions of test virus.				
	Undiluted.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
"W.S."					
Normal	..	..	$4^5, 4^5, 4^5$	$4^6, 4^6, 4^7$	3, 3, 3
Immune	$4^6, 3, 1$	$4^5, 0, 0$	..	..	..
"Melbourne"					
Normal	..	..	$4^5, 4^6, 4^7$	$4^6, 4^6, 3$	3, 3, 2
Immune	$4^5, 3, 2$	..	$4^7, 2, 1$	3, 1, 0	..
"Philadelphia"					
Normal	..	..	$4^6, 4^6$, X	$4^6, 4^6, 4^7$	3, 3, 2
Immune	$4^4, 4^4, 3$	..	0, 0, 0	2, 2, 0	..
"P.R. 8"					
Normal	..	..	$4^5, 4^5, 4^7$	$4^5, 4^6, 4^7$	3, 2, 2
Immune	$4^4, 4^5, 4^5$	..	3, 2, 1	2, 2, 1	..
"Leningrad"					
Normal	..	..	$4^5, 4^5, 4^7$	$4^6, 4^7, 3$	$4^6, 4^7, 3$
Immune	$4^6, 4^6, 4^7$	$4^6, 4^6, 0$	..	..	..

After the test inoculum the mice were kept in individual glass jars for 6 to 7 days. Mice dying were examined, and if the lungs showed complete or almost complete consolidation they were regarded as specific deaths. All surviving mice were killed either on the sixth or seventh day, and the extent of consolidation recorded according to the convention used in previous work. Not infrequently lungs showed varying degrees of congestion but not consolidation; these were regarded as negative, only definitely consolidated areas being recorded.

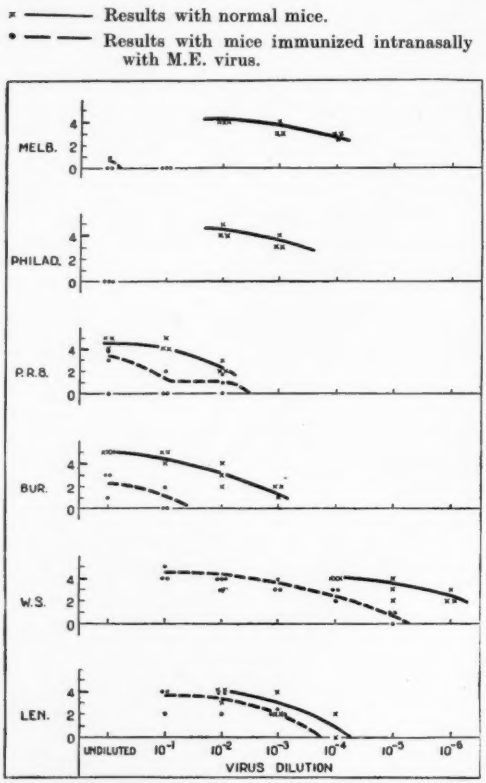

FIG. 1.—The resistance of mice immunized with living M.E. virus against infection with different strains of mouse-pathogenic influenza virus. Ordinates represent the intensity of lesions graded as follows: 0, No lesions in the lungs. 1, Small lesions involving less than one-sixth area of the posterior lung surface. 2, Lesions involving one-sixth to one-half lung area. 3, Lesions involving one-half to seven-eighths lung area. 4, Mice dying with completely consolidated lungs in 5 to 7 days. 5, Mice dying within less than 5 days.

Five complete comparisons of the action of the mouse strains "Melbourne" and "W.S." on M.E. immune mice were carried out. In every series immunity against "Melbourne" was almost absolute, a few small patches of consolidation occasionally being observed with undiluted test virus. Higher dilutions were completely negative. With strain "W.S." an interesting difference was shown in the response of mice immunized (*a*) with a large (lesion-producing) dose of M.E. virus at a time when this strain was producing a fairly heavy mortality in mice, and (*b*) with a small (1 : 100) dose of the same virus or with a larger (undiluted) dose of M.E. when it was producing only minimal lesions. The first group of mice were considerably more resistant to "W.S." than those in which no lesions were produced by the immunizing infection. Table I illustrates the results of comparing two groups of mice immunized respectively with undiluted and 1 : 100 M.E. virus in its active phase. It may be noted that there was no difficulty in distinguishing the grey, usually sunken, old lesions produced by the original inoculation from the areas of consolidation produced by the test infection.

Only the latter are recorded. If regard is had only to the deaths and survivals the difference in the two groups toward " W.S." is particularly evident.

Tests of the immunity of mice immunized without obvious lesions against the mouse strains " W.S.", " Melbourne ", " P.R. 8 ", " Philadelphia' , " Leningrad " (Smorodintseff) and " Bur." (Stuart-Harris *et al.*, 1938) are represented graphically in Fig. 1. It will be seen that immunity against " Melbourne " and " Philadelphia " is complete, in agreement with all our previous studies on the serological relationship between these two strains. " W.S." and " Leningrad " are almost as active in immune mice as in the normal control animals, while " P.R. 8 " and " Bur " occupy an intermediate position. All these experiments have been repeated with similar findings.

In order to obtain mice immunized with strain " W.S." by the intranasal route it was necessary to inoculate them *unanæsthetized*, since no non-virulent egg-passage strain is available. Of 50 mice inoculated 3 died with specific lung lesions. Twelve days later the survivors were re-inoculated under anæsthesia with " W.S." virus diluted 1 : 100 and all survived. The test inoculations were made a fortnight later. The results given in Table II are much less distinct than those in the M.E. experiments. There is obviously an increased resistance against all strains, and an indication that the immunity is most active against the homologous virus. It is not, however, complete, and amongst the other strains there is no division into the antigenic groups differentiated by serological tests. The strains " Melbourne " and " Philadelphia " are actually more effectively protected against than " Leningrad ", which serologically is much closer to " W.S."

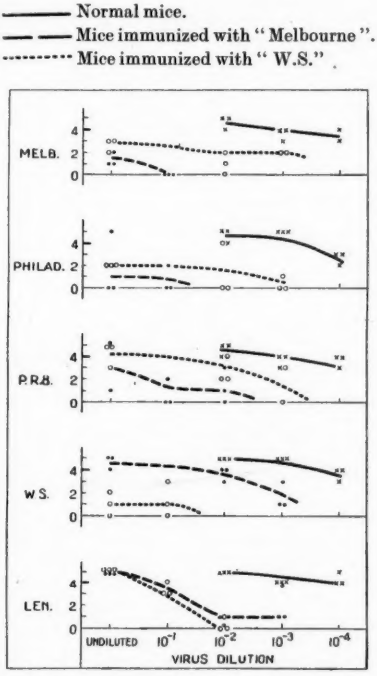

FIG. 2.—Resistance of mice immunized intraperitoneally with strains " Melbourne " and " W.S." against intranasal administration of different strains. General arrangement as in Fig. 1.

A further series of experiments was made to compare the resistance to different strains of mice immunized by intraperitoneal injection of mouse strains of " Melbourne " and " W.S." Two-day lungs were used for preparing the emulsion, 1 lung to each 4 c.c. of broth, and 0·25 c.c. was given intraperitoneally on two occasions 12 days apart. Tests of immunity were made 10 days later, with the results depicted in Fig. 2. The findings are fairly comparable with those of the two other experiments in showing a more specific immunizing action of " Melbourne " than of " W.S."

DISCUSSION.

The question of antigenic differences amongst human influenza strains is still under discussion. The differentiation into two main antigenic types which has been proposed (Burnet, 1937*b*) will probably be found inadequate when further strains are available, particularly in the light of the brief report on the strains isolated in the 1937 English epidemic by Stuart-Harris *et al.* (1938). The most prevalent type in that epidemic appears to fall in the " New World " group, but other strains were found to be quite distinct from this and from " W.S.", etc. The main result of the present work has been to show that immunization of mice with " Melbourne " egg virus given intranasally, while highly effective against the homologous strain and the closely related " Philadelphia ", produces only slight resistance to " W.S." and " Leningrad " and a moderate resistance to " P.R. 8 ". Immunity induced by inoculation of " W.S." intranasally without anæsthesia or intraperitoneally is of more generalized character.

SUMMARY.

1. A relatively specific immunity can be induced in mice by the intranasal inoculation of " Melbourne " egg-adapted influenza virus. Protection against the strains " Melbourne " and " Philadelphia " is complete, " P.R. 8 " occupies an intermediate position, and very little resistance against " W.S." and " Leningrad " is induced.

2. Mice can be immunized effectively against a virulent (" W.S.") strain by intranasal administration of living virus in unanæsthetized mice.

3. Immunity induced by either intranasal or intraperitoneal inoculation of " W.S." is relatively generalized.

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SURFACE FILMS OF ANTIBODIES AND ANTIGENS.—I. THE EFFECT OF SPREADING ON A WATER SURFACE ON THE SPECIFIC PROPERTIES OF PNEUMOCOCCUS (TYPE II) ANTIBODY AND HORSE-SERUM GLOBULIN.

J. F. DANIELLI, MARY DANIELLI, AND J. R. MARRACK.

From the Department of Physiology and Biochemistry, University College, London, and the Hale Clinical Laboratory, The London Hospital.

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AT an air-water or an oil-water interface proteins spread into a monolayer approximately one polypeptide residue (9 Å.) thick. The behaviour of these films has been summarized by Mitchell (1937) and Langmuir (1938) in the case of the air-water interface, and in that of the oil-water interface by Danielli (1938). The unfolding of the molecule that occurs at these liquid interfaces is usually accompanied by the loss of the specific properties of the protein concerned. On the other hand at a liquid-solid interface, where adsorption of the protein occurs at a solid surface, this unfolding does not occur, the molecule retaining its original form and specific properties. Thus a protein adsorbed on collodion or aluminium hydroxide retains its specific power of combining with antibody or antigen (Jones, 1927, 1928; Freund, 1930), and may even be recovered unchanged. Shaffer and Dingle (1938) have allowed egg albumen to adsorb on solid stearate surfaces, and shown that the adsorbed molecules combine specifically with the corresponding antibody.

Presumably a film formed by adsorption on a *solid* film at the air-water interface would also retain its specificity. Such films will be the subject of later papers.

Whether or no an antibody protein spread on a surface in the unrolled form would retain its ability to combine with antigen would depend on the nature of the receptor groups of the antibody; in the same way the ability of an unrolled protein antigen to combine with antibody would depend on the nature of the combining groups of the protein.

We have made experiments of two types: (1) Purified antipneumococcal antibody was spread on water and the homologous polysaccharide was added to the underlying solution; (2) antigen (horse-serum globulin) was spread on the water surface and antibody (rabbit serum) was added to the underlying solution. It was essential that relatively pure preparations of proteins should be used for spreading. If, for example, the proteins of antiserum, only one-tenth of which were antibody proteins, were spread on a water surface, any reaction between the antibody on the surface and antigen in the bulk of the fluid would quite possibly escape detection.

EXPERIMENTAL DETAILS.

Measurements of the surface pressure and surface potential at the air-water interface were made with the customary apparatus (Adam, 1938). Surface pressure at the oil-water interface was measured by the method of Askew and Danielli (1936).

The antibody to Type II pneumococcus was prepared by a method resembling that of Horsfall and Goodner (1937). The precipitate, formed after the addition of rather less than the equivalent amount of polysaccharide to antiserum, was washed with 1 per cent. NaCl solution and then stirred with 10 per cent. NaCl solution at 37° C. for one hour. The filtered supernatant fluid was dialysed for 48 hours against running tap-water. The precipitate which formed on dialysis was extracted with 1·6 per cent. NaCl solution and the extract centrifuged after keeping for two days in the refrigerator. Over 90 per cent. of the protein in this extract was precipitable by Type II polysaccharide.

The Type II polysaccharide was prepared by the method of Felton, Kauffman and Stahl (1935). It contained no substances which formed films at an air-water interface.

Horse-serum globulin was prepared by dilution of serum with two volumes of water and the addition of three volumes of saturated $(NH_4)_2SO_4$ solution. The precipitated globulin was centrifuged down, washed with half saturated $(NH_4)_2SO_4$ and dialysed against 1 per cent. NaCl solution. Rabbits were immunized against this globulin by repeated intraperitoneal injection. The optimum proportions of the serum used were 0·92 mg. of horse globulin to 1 ml. of serum.

For use at the air-water interface the solutions of pneumococcus antibody and the serum globulin in 1·6 and 1·0 per cent. NaCl solution respectively were evaporated to dryness in vacuo after freezing. The powders so obtained exhibited high activity if redissolved.

RESULTS.

These experiments were begun two years ago with antibody concentrated by the Felton method. The method of extraction of Heidelberger and Kendall (1936) gives a much purer product, and only the experiments with antibody prepared in this way are reported here.

The type of result obtained at the air-water interface is illustrated by Fig. 1. The circles are for a film of Type II pneumococcus antibody two hours after spreading from particles of the solid dropped on to the surface of a 1 per cent. NaCl solution, pH 7·2, at 20° C. The antibody spread rapidly and formed a stable elastic solid film, tending to increase slowly in area. Immediately after the measurement recorded in the figure specific polysaccharide was added to the underlying solution to bring its concentration up to 1 part of polysaccharide in 500,000, and the solution well mixed. The crosses on the diagram represent readings taken 15 minutes after the circles. It will be seen that there was no significant difference between the two sets of points. A

number of variants on this type of experiment were tried ; *e. g.* the antibody was spread on a solution already containing polysaccharide : the experiments were repeated at 0° C. ; the film was allowed 24 hours to settle down after spreading ; in no case was there any difference between the film in the presence or absence of the polysaccharide when films of the same age were compared.

The surface pressure measurements were repeated at the oil-water interface, though less extensively, and with the same result.

With the horse globulin and rabbit antiserum measurements of surface potential only were made. A globulin film was spread from the solid on the

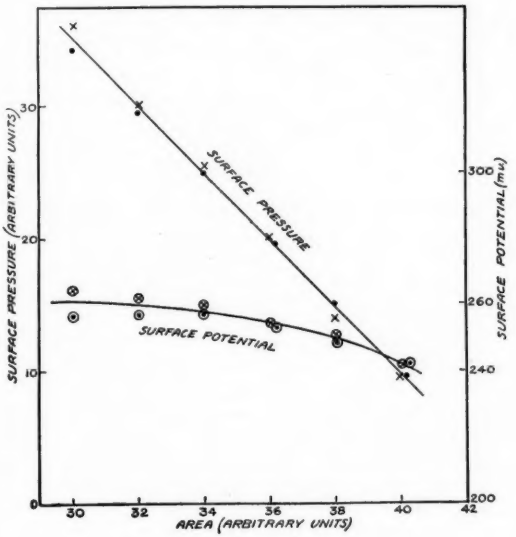

FIG. 1.

surface of 25–50 c.c. of 1 per cent. NaCl, pH 7·2, contained in a waxed vessel. The film, which spread very rapidly, was an elastic solid and had a surface potential of about 200 mv., which decreased slowly with time. At various intervals after spreading, commencing 10 minutes after spreading, serum was injected under the films. Varying amounts of serum were used, up to 1 c.c. in 25 c.c. NaCl solution. After the injection of serum the surface potential of the film was in no case changed by more than $\pm$ 3 mv. It thus seems improbable that any reaction involving polar groups occurs between the film and serum. It is possible that a certain degree of penetration of the film by serum protein molecules occurred, since there was little difference between the surface potential of the horse globulin and the protein film formed by adsorption from the serum.

DISCUSSION.

Any reaction between film and substrate involving polar groups would be reflected in a change in surface potential. Simple penetration of the film by the substrate would involve changes in surface pressure, and possibly in surface potential also. Neither change occurred with pneumococcus antibody, so that it is safe to say that in the unfolded state of the molecule produced by spreading at a liquid interface those specific properties which lead to the antibody-antigen reaction have been almost, if not entirely lost.

In the case of horse-serum globulin it is equally certain that no polar group reaction takes place, but it is possible that penetration of the film has occurred ; it is highly improbable, however, that this alone would be sufficient to account for the antibody-antigen reaction.

These experiments suggest that the receptor group of Type II pneumococcal antibody and the combining group of horse-serum globulin are not prosthetic groups, nor combinations of amino acids which retain the arrangement conferring the specific properties when the protein is unrolled.

SUMMARY.

Surface films of pneumococcus (Type II) antibody spread at the air-water and oil-water interfaces show no sign of ability to combine with the specific polysaccharide. Surface films of horse-serum globulin show no signs of ability to combine with a highly active rabbit antiserum.

One of us (J.F.D.) is indebted to the Department of Industrial and Scientific Research for a Senior Research Award. The experiments at the oil-water interface were carried out in collaboration with Dr. F. A. Askew. We are indebted to Prof. N. K. Adam for the loan of the oil-water interface apparatus, and to the Wellcome Physiological Research Laboratories for a supply of Type II antipneumococcal serum.

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A PHOTO-ELECTRIC STUDY OF REACTIONS BETWEEN DIPHTHERIA TOXIN AND ANTITOXIN.

C. G. POPE AND M. HEALEY.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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THE flocculation which occurs between diphtheria toxin or toxoid and antitoxin when mixed in suitable proportions was made the basis of an *in-vitro* method for determining the strength of toxin or antitoxin by Ramon (1922). This method has been used extensively in various laboratories, and although there have been various reports of cases of non-specific flocculation, Glenny and Okell (1924), Glenny and Wallace (1925), Maloney and Weld (1925), Schmidt (1926), and of sera having abnormal *in-vivo*/*in-vitro* ratios, Glenny and Okell (1924), Glenny, Pope, Waddington and Wallace (1925), Sordelli and Serpa (1925), Moloney and Weld (1925), Barr and Glenny (1938), little work has been done on the physical conditions underlying this reaction.

It is known that the mixture which gives the greatest mass of floccule precipitate is not the first flocculating mixture, and no reason has been advanced to explain this. It appeared possible that by studying the earlier stages of the flocculation reaction more detailed information might be gained, and since it is known that flocculation is preceded by the production of opacity, a photo-electric method has been used to study this part of the reaction. Although this method has given a great deal of additional information regarding the reaction, it has still left many points of interest undecided.

EXPERIMENTAL.

Apparatus.

The photo-electric apparatus used was constructed from two photo-electric cells of the selenium type. The light source was a 6-volt spot-light bulb, and a divided light path was arranged so that one beam passed through the long cell containing the solution before falling on one photo-cell, and the other fell directly on the second photo-cell, before which was placed a shutter controlled by slow-motion gear. The two photo-cells were connected in opposition, and the current generated was measured by a galvanometer giving a deflection of 1500 mm. per microampere. The method is shown in the diagram.

The shutter was a circular metal disc with the spindle mounted eccentrically, so that by slow rotation the amount of illumination on the second cell could be accurately adjusted. Thus, with any liquid in the glass cell, the shutter could be adjusted so that the galvanometer reading was zero (or any other scale reading that was desired for an experiment).

In this form the apparatus was very sensitive to small changes in the optical condition of the liquid in the glass cell, and for work with various mixtures of toxin and antitoxin it proved fairly satisfactory. It suffered from the defect that its absolute sensitivity varied with the initial colour of the toxin used, and it was difficult to make any absolute form of calibration. The glass cell required 30 ml. of solution for each observation.

Much of the work was done with toxins or toxoids partially purified by charcoal absorption of coloured materials followed by ultra-filtration and washing. This treatment gave almost colourless materials, and made more easy the study of the opalescence produced by the interaction of the toxin and

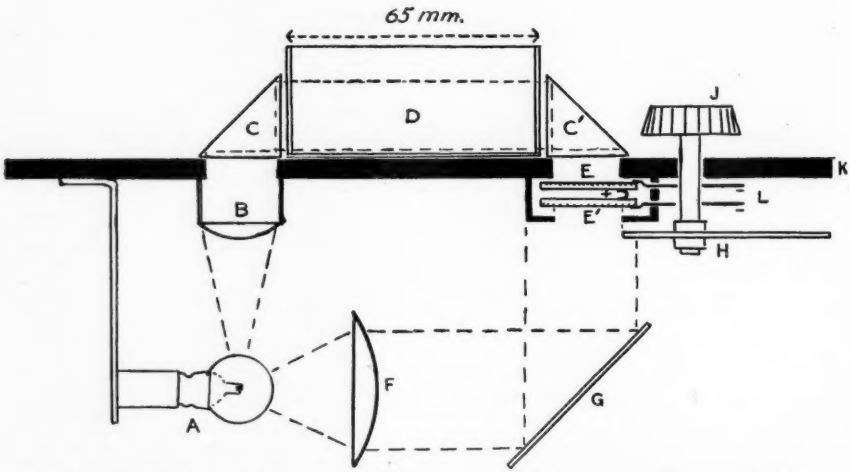


FIG. 1.—Diagram of the photo-electric apparatus. A. Six-volt lamp. B. Lens. C and C^1. Prisms. D. Glass cell. E and E^1. Photo-electric cells in opposition. F. Lens. G. Mirror. H. Eccentric shutter. J. Shutter control. K. Ebonite panel. L. Leads to galvanometer.

antitoxin. Where it was not possible to purify the initial toxin, it was diluted 1 in 4 or more with saline, so that it contained about 8 Lf. units per ml. The antitoxic sera used were all purified, either by the routine ammonium sulphate method, or by a new method (Pope, 1938), and had all been passed through Berkefeld candles. Although not quite optically clear, the small amounts of serum used did not introduce any marked opacity in control dilutions of broth or saline.

Early in this work it became clear that the mixture containing toxin* and antitoxin in optimal (first flocculating) proportions developed its opacity at a greater rate than mixtures containing more or less antitoxin. This could be made the basis of a method for determining the titre of toxin or toxoid, but the labour involved in a determination in this way is very great, and it appears

* For the *in-vitro* work described in this paper the word "toxin" should be read as "toxin or toxoid", since the reactions of either were similar.

to offer no advantage over the simple technique of observing the first visible flocculating mixture. Some observations on the relative rates at which opacity developed in mixtures are given later, but for the greater part the observations here recorded deal with special aspects of the reaction between toxin and antitoxin.

The Rate of Development of Opacity in a Toxoid Following the Addition of One Equivalent Dose (in vitro) of Antitoxin.

The equivalent dose of antitoxin is the volume of serum which contains the number of *in-vitro* units equal to the number of toxin units (Lf). This produces the mixture which flocculates more rapidly than any other.

The toxoid (RX 3230) containing 16 Lf units per ml. was measured (30 ml.) into the glass cell and the galvanometer brought to balance by means of the adjustable shutter.

TABLE I.—*Rate of Production of Opacity in Equivalent Mixtures of Diphtheria Toxin and Antitoxin Prepared in Different Ways.*

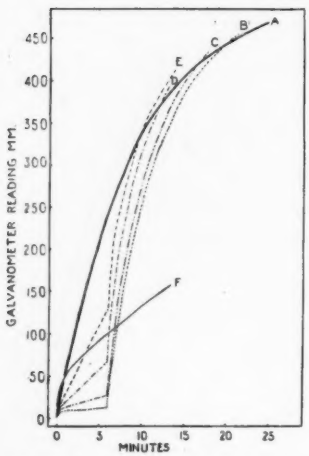
Materials: Toxoid RX 3230 DP 89 16 Lf units/ml. (Pope, 1938.) Antitoxin					
First dose of antitoxin	50%	60%	70%	80%	100%
Second dose of antitoxin	50%	40%	30%	20%	—
Time (minutes).	Galvanometer readings in mm.				
0·5	8·5	11·5	13	17	27
1	9·5	13·5	18·5	27	49
2	10	16·5	29	49	96
3	11	19·5	38·5	69·5	140
4	12	21·4	48	90	178·5
5	12+	24	57·5	109	214
6	A	A	A	A	246·5
6·5	61	89	141	199·5	—
7	98	127	181	232	274
8	161	191	240·5	280	298·5
9	213	240	284·5	315	321
10	256	281	319·5	343	341
11	292	313·5	346·5	366·5	356·5
12	322	341	369·5	385·5	371
13	347	364	388	402	384
14	368·5	383	403	416	395
15	386·5	400	..	..	..
16	402·5	414·5	..	..	..
17	416	426	..	..	..
18	427	437	..	..	433
19	437·5	..	..	..	..
20	446·5	..	..	..	445
21	454	..	..	..	452
22	..	..	..	..	457
25	..	..	..	..	469

A. Time of second addition of antitoxin.

See Graph I.

The antitoxin (DP 89) diluted to contain 1300 units per ml. was measured (0·375 ml.) with a micrometer syringe (Trevan, 1925) into a glass spoon. At the time of addition of the antitoxin to the toxoid a stop-watch was started, and the galvanometer deflection was read at intervals from 0·5 minutes onwards.

From the figures in the fifth column of Table I it will be seen that the mixture steadily increased in opacity, rapidly at first and later more slowly. The other figures in the remaining columns show for a period up to 5 minutes the rates for mixtures having less than one equivalent of antitoxin. For a reaction time of 5 minutes the optimal mixture showed a deflection of 214 mm., while the mixture having 20 per cent. less antitoxin showed only 109 mm. The rate also diminishes when more than one equivalent of antitoxin is added. Table II (last column) shows the rate for a two-equivalent mixture, and it will be seen that at 5 minutes the deflection was 92·5 mm. (These results are shown in Graph I.) Mixtures which contain up to about 2·0 equivalents of antitoxin, although they show a slower rate than the equivalent mixture, ultimately develop greater opacity. Marrack and Smith (1930), Healey and Pinfield (1935) have shown such mixtures have a greater floccule nitrogen content than those at one equivalent. It is well known that the time of flocculation (Kf) depends, amongst other things, on the toxins and antitoxins used, and our observations have shown that the rate of opacity production varies similarly. Table I (column 5) can be compared with Table III (column 1 up to 5 minutes); they show the rate for the new process antitoxin (Pope, 1938) and that for an ammonium sulphate concentrated product, both with the same toxoid. Antitoxin concentrated by the new process flocculates very rapidly with toxins, and Table I shows that the rate at which opacity is produced is very much greater than for the ammonium sulphate product.



GRAPH I.—Showing the increase of opacity with time. Curve A, 100% antitoxin direct. Curve B, 50% followed by 50%. Curve C, 60% followed by 40%. Curve D, 70% followed by 30%. Curve E, 80% followed by 20%. Curve F, 200% antitoxin direct.

Having studied the results obtained from an addition of the antitoxin in one dose, it became a matter of interest to study the results of adding the same total amount of antitoxin, but added in fractional doses. In this series of experiments we added the first part, from 50 to 80 per cent. of the total amount, and at the sixth minute added the residue to make 100 per cent. The results (Table I) show that a small dose of antitoxin of the order of 50 per cent. of the total amount produced but a small increase in opacity, and that the rate of opacity production increased with increase in the percentage amount of antitoxin added in the first dose. Following the second addition the rate of reaction was very high, and it will be seen from Graph I that the curves cross

that for the mixture made directly, the change being a progressive one, as the first fraction of antitoxin is increased from 50 to 80 per cent. This suggested that such mixtures, and particularly the 80/20 per cent. one, should flocculate in less time than the control mixture. The point was investigated, and the results (not given here in detail) showed that such mixtures did in fact flocculate in less time than the control when tested by the usual flocculation procedure.

The Rate of Development of Opacity in an Equivalent Mixture to which further Additions of Antitoxin were made.

The materials and general conditions were as previously described. After allowing the balanced mixture to react for 6 minutes, more antitoxin was added (10–100 per cent. additional antitoxin) and the reaction changes followed. From the figures in Table II it will be seen that the normal course of the reaction was altered, the most rapid increase in opacity being that for a second addition of antitoxin equal to the equivalent dose, *i. e.* a total of 200 per cent. antitoxin in the reacting mixture. This is shown from the results in Table II for a reaction time of say 10 minutes, where the values are :

Control 341 mm., 10 per cent. 379 mm., 20 per cent. 402·5 mm., 40 per cent. 433·5 mm., 60 per cent. 466·5 mm., 80 per cent. 489 mm., 100 per cent. >500 mm.

TABLE II.—*The Addition of Antitoxin to Unflocculated Equivalent Mixtures.*

	Materials : Toxoid Antitoxin RX 3230 DP 89 16 Lf units/ml. (Pope, 1938.)							
First dose of antitoxin	100%	100%	100%	100%	100%	100%	100%	200%
Second dose of antitoxin	..	10%	20%	40%	60%	80%	100%	—
Time (minutes).	Galvanometer readings in mm.							
0·5	27	30	25	28	31	30	29·5	39
1	49	52	45·5	49·5	53	55	53	49
2	96	96	88	93	100	102·5	100	63·5
3	140	138·5	127·5	136	143	147·5	143	74·5
4	178·5	177	163·5	173	183	187·5	183	84
5	214	211·5	197	207	217·5	223	218	92·5
6	246·5	A	A	A	A	A	A	101·5
6·5	..	285	297	339	386·5	407	416	—
7	274	304	322	364	406·5	435·5	444	110
8	298·5	334	356	394	435·5	462	470	118
9	321	359·5	381	416	453	477·5	485	125·5
10	341	379	402·5	433·5	466·5	489	..	133
11	356·5	397	420·5	449	478·5	..	..	140
12	371	412·5	435	461·5	488	..	..	146
13	384	426	447·5	473	..	..	..	153·5
14	395	437·5	459	484	..	..	..	166
63	..	..	..	..	..	..	..	411·5

A. Time of second addition of antitoxin.

In contrast to these results are the figures in the last column of Table II, where 200 per cent. of antitoxin was added in a single dose. The rate of reaction in a mixture produced in this way was very slow, *i. e.* at 10 minutes the deflection produced was only 133 mm.

For reasons which will be discussed later the remaining experiments in this section were made with the same toxoid, RX 3230, and antitoxin concentrated by the ammonium sulphate process.

TABLE III.—*The Addition of Antitoxin to Unflocculated Equivalent Mixtures.*

Materials :	Toxoid RX 3230 Antitoxin PC 2325			16 Lf units/ml. (Ammonium sulphate concentration).			
First dose of antitoxin	100%		100%		100%		100%
Second dose of antitoxin	50%		100%		150%		200%
Time (minutes).	Galvanometer readings in mm.						
0·5	9·5		9·5		12·5		9
1	16·5		16·5		21		16
2	28·5		28		33		26·5
3	38		36·5		43		36
4	46·5		45		52·5		44·5
5	54·5		53		61		52·5
6	A		A		A		A
6·5	100		113·5		133		126
7	111		126		143·5		137
8	125		132·5		153		145·5
9	134		144		158		148
10	141·5		149		159·5		149·5
11	148		152		161		149·5
12	154		155		162		148·5
13	159		158·5		162		148
14	165		161		162		147
15	..		..		..		146
16	..		..		..		145
17	..		..		..		144
18	..		..		..		143
19	..		..		..		142
20	..		..		..		141
	F.		N.F.		N.F.		N.F.

A. Time of second addition of antitoxin.
F. Indicates that the mixture would flocculate.
N.F. Indicates that the mixture would not flocculate.

Equivalent mixtures were made from RX 3230 and PC 2325, and after an interval of 6 minutes we added further amounts of this antitoxin equal to 50, 100, 150 and 200 per cent. of the equivalent dose. The antitoxin used had a lower value in units per ml. than the purified antitoxin of the previous experiment, and also was more coloured. We therefore determined the correction to be applied to the galvanometer readings due to non-specific effect

of the serum, by adding various amounts of it to a non-reactive diluted broth which was interchangeable in the colorimeter cell with the toxoid used (RX 3230). The corrected readings are given in Table III, and they show the sudden increase in opacity for all these additions of antitoxin, reaching a maximum for the addition of 150 per cent. excess antitoxin over the equivalent dose. With the addition of 200 per cent. antitoxin there was an initial rise in galvanometer readings, but after about 12 minutes the readings fell very slowly and regularly, indicating diminishing opacity. This result is much more clearly shown in the next experiment.

In this experiment the time interval between making the additions of antitoxin was extended from 6 minutes to 38 minutes. At this point 200 per cent. excess of antitoxin was added. The readings have been corrected as described and are given in Table IV and Graph II. They showed a similar but more marked sudden increase in opacity following the antitoxin addition, and after 44 minutes the opacity fell sharply at first and then more slowly. In order to determine whether this result was a specific one due to antitoxin, the experiment was repeated by allowing an equivalent mixture to react for 38 minutes, and then adding a volume of concentrated scarlet fever antitoxin equal to that of the 200 per cent. dose of diphtheria antitoxin. Beyond a small increase in galvanometer reading due to the colour of the serum, this material produced no effect.

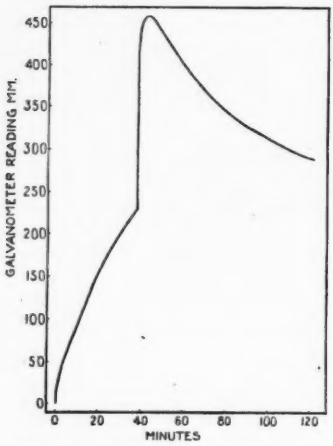
TABLE IV.—*The Addition of Antitoxin to Unflocculated Equivalent Mixtures.*

Materials: Toxoid. RX 3230 16 Lf units/ml.
Antitoxin PC 2325 (Ammonium sulphate concentration.)

Time (minutes).	Galvanometer readings in mm.	Time (minutes).	Galvanometer readings in mm.
0	1 equivalent antitoxin added	..	..
0·5	11·5	45	405
1	19·5	46	402
2	31·5	47	398·5
3	41·5	48	395
4	50·5	49	392
5	58·5	50	388
10	94·5	51	384·5
15	125	52	381
25	176·5	53	377
30	198·5	54	373
35	218·5	55	369
38	2 equivalents antitoxin added	56	365·5
		57	361
38·5	359	58	357·5
39	382	59	354
40	399	60	350·5
41	405	90	272
42	408·5	122	241
43	408·5	152	223·5
44	407·5	172	216
		Left overnight	172

See Graph II.

From these experiments it was obvious that the time interval between making the antitoxin additions affected the results obtained, and in another experiment this point was investigated further.



GRAPH II.—Showing the effect on opacity of one equivalent dose followed after 38 minutes by a further two equivalents of antitoxin (see also Table IV).

A bulk of an equivalent mixture was made from a routine toxin (BHVT 392 diluted to 8 Lf units per ml.) and PC 2325 (1175 antitoxic units per ml.) and kept at room temperature. At various intervals 30 ml. of this mixture were placed in the glass cell, and the shutter adjusted to bring the galvanometer to zero. Sufficient antitoxin (0·20 ml. measured with a micrometer syringe) was added on each occasion to make the final antitoxin equal to two equivalents. The effect produced in each case was measured, and it was found that as the time interval between the addition of the first equivalent dose and the second equivalent of antitoxin increased, so did the reaction increase, up to a time interval of 160 minutes. Between this point and that of the mixture kept at room temperature overnight there was no further increase in reactivity. The mixture kept overnight had flocculated, and was shaken to redisperse the floccules before adding extra antitoxin. These figures are recorded in Table V.

TABLE V.—*The Addition of a Second Equivalent of Antitoxin to an Equivalent Mixture at Various Times after Mixing.*

Materials: Toxin BH VT 392 diluted to 8 Lf units/ml.
Antitoxin PC 2325 (Ammonium sulphate concentration.)

Time after mixing (minutes).	5	10	20	40	80	120	160	Overnight.
Time after addition of second equivalent (minutes).	Galvanometer readings in mm.							
0·5	81	80	111	154	195	218	233	223
1	97	103	136·5	189	244	272	287	278
2	114	122·5	162	221	285	321	334	329
3	123	136	175	238	307	346	358	355
4	..	145	184·5	249	321	361	374	370
5	..	151	194	258	331·5	372	386	382
6	..	156·5	199	265	340	380	393	390

The Opacity of Mixtures of Toxin and Antitoxin of Varying Composition at Equilibrium.

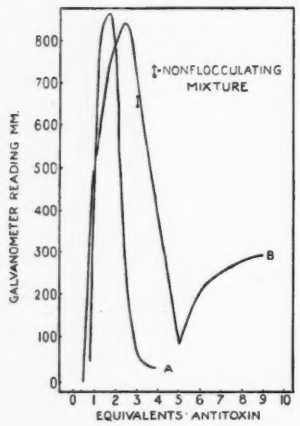
The general experimental method employed was as follows: Constant volumes of toxoid were measured out and antitoxin added to form a range of mixtures from about 0·5 to as much as 9·0 equivalents. These mixtures in stoppered bottles were placed in a bath at 45° C., generally for 7 hours, after which they were left at room temperature overnight. After thorough shaking, a 1 in 4 dilution was made with saline and the density of the mixture measured on the instrument. Usually the most dense of the mixtures forming the series was placed in the cell and the shutter adjusted to give a reading of about 400 mm. The less dense mixtures gave, with this arrangement, readings in the reverse direction, and these were made positive readings by reversing the leads to the galvanometer. The readings were then all corrected so that the least dense mixture had a reading of zero and all other readings were positive to this. It must be pointed out that the heights of the curves in Graphs III and IV are not directly comparable owing to the absence of a true base-line. It is rather their general shape and width in relation to the amount of antitoxin present that is important.

The results with two toxoids (RX 3230 and RX 3304) are reported here. The first of these was partially purified as already described, but it contained two antigen systems, one true diphtheria toxoid, and the other a form of bacillary antigen. The reactions between this antigen and antitoxic sera will form the subject of another paper and will not be considered in detail here. The other toxoid had been specially purified, and contained approximately 2000 Lf units per mg. of heat-coagulable protein nitrogen. This figure is in good agreement with the results of Eaton (1936) and Pappenheimer (1937) for purified diphtheria toxin.

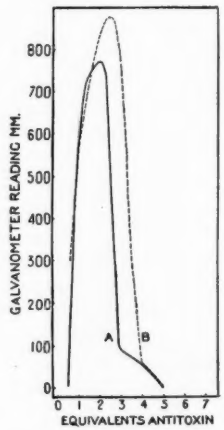
The sera used were PC 2325 (ammonium sulphate concentration) and DP 6K and DP 89 (both purified by the new process) (Pope, 1938). With mixtures of RX 3230 and PC 2325 the limits of flocculation were exceptionally narrow; the mixture containing 0·75 equivalents did not flocculate, nor did that containing 1·75 equivalents. With the same toxoid and DP 6K serum, all mixtures from 0·5 to 9·0 equivalents showed flocculation except for the mixture containing 3·0 equivalents of antitoxin. In this series of mixtures there were two zones of flocculation, with a non-flocculating range between them, and the 3·0 equivalent mixture formed part of this non-flocculating zone. Where a non-flocculating mixture occurred in a series such as this, the galvanometer readings gave no indication of this state; such mixtures show a density greater than that of the equivalent mixture. Graph III shows the galvanometer deflections produced by the opacity of mixtures prepared from toxoid RX 3230 and antitoxins PC 2325 and DP 6K. The curves show that maximal density was obtained with PC 2325 at 1·75 equivalents and at 2·5 equivalents with DP 6K. It is also clear from the curves that these two types of antitoxins show different reactivities against the same toxoid, that for PC 2325 being much narrower and showing no evidence of interaction with the second antigen which was known to be present, whereas DP 6K shows a broader zone and evidence for reaction with the second antigen.

Graph IV shows the results with RX 3304 (which we believe to be almost pure diphtheria toxoid). Here again the maximal densities were at 2·0 and 2·5 equivalents for PC 2325 and DP 89 respectively, and DP 89 showed a broader zone of reactivity. There was, however, no indication here of the double zone with the DP material, and exhaustive tests with more antitoxin failed to show any secondary reaction.

These mixtures have been considered as being in a state of equilibrium for the following reason: Similar mixtures to those recorded were prepared in



GRAPH III.—Showing the equilibrium opacities of mixtures made from PC 2325 (curve A) and DP 6K (curve B) against toxoid RX 3230.



GRAPH IV.—Showing the equilibrium opacities of mixtures made from PC 2325 (curve A) and DP 89 (Curve B) against toxoid RX 3304.

an indirect manner by first forming an equivalent mixture and allowing it to flocculate, then adding either toxoid or antitoxin to convert portions of it to form a similar series of mixtures having varying equivalents of antitoxin. These, after heating in the water-bath, were diluted as before and their opacity compared. The results were of the same type as shown in Graphs III and IV, and the manner of preparation of these mixtures appeared to be immaterial.

DISCUSSION.

Using this photo-electric method to study the early stages of the reaction between diphtheria toxin and antitoxin, we have found that the equivalent mixture is the one which develops opacity at the greatest rate. Mixtures made with either more or less antitoxin than the equivalent amount show slower rates. The rate is affected more markedly by a reduction in the amount of antitoxin than by an increase, and the ultimate opacity produced is usually greatest at 2·0–2·6 equivalents of antitoxin (depending on the material used).

The equivalent mixture can therefore be detected before visible flocculation takes place, but this method is not suggested in place of the simple flocculation technique.

Where the equivalent mixture is made fractionally, we have shown that following the second addition of antitoxin, the rate of opacity production may be greater than in the control mixture. This suggested that such fractionally made mixtures might flocculate in less time than the control, and it was found that this was true. The reason for this will be dealt with later in the discussion.

Healey and Pinfield (1935) investigated the combination between toxin and antitoxin, using the *in-vitro* method to determine the reactions taking place, and have shown that after combining to form the equivalent mixture, the toxin-antitoxin complex can take up further antitoxin to the extent of approximately another equivalent. They expressed the reactions as—

$$\mathrm{T} + \mathrm{A} = \mathrm{TA},$$
$$\mathrm{TA} + \mathrm{A} = \mathrm{TA_2},$$
$$\mathrm{TA_2} + \mathrm{T} = 2\,\mathrm{TA},$$

and showed that TA_2 was formed either if all the antitoxin (two equivalents) was added at once, or if TA was first formed and allowed to flocculate before adding the second equivalent. They also showed from nitrogen determinations that the mass of floccules increases as the composition changes from TA to TA_2. Since these observations were made (on unconcentrated and ammonium sulphate concentrated antitoxins) a new method (Pope, 1938) for purification of antitoxin has been used, and it has been found that the product so obtained differs in certain properties from the antitoxins used by Healey and Pinfield. Some of the differences are given below. For convenience the new product is called (*a*), and the ammonium sulphate product (*b*).

(1) The time of flocculation (Kf) with antitoxin (*a*) is far less than with (*b*).

(2) The antitoxin-toxin combining ratio is 2·6–2·8 for (*a*), whereas Healey and Pinfield showed it was generally 2·0 for (*b*).

(3) Flocculation is not inhibited until more than three equivalents of (*a*) are present, whereas more than two equivalents of (*b*) are generally inhibitory.

Because of these differences both types were used in the experimental work.

In Table I (column 1) the rate of opacity production where antitoxin of type (*a*) was used was much greater than for antitoxin type (*b*), Table III (readings up to 5 minutes), both against the same toxoid. This is in good agreement with the fact that type (*a*) flocculates more rapidly than type (*b*).

The interaction of additional antitoxin with an equivalent mixture has been shown to be greatly influenced by the conditions existing in the mixture at the time of the second addition. In Table II (column 8) we show that when two equivalents of antitoxin were added at once, 63 minutes were required for the production of opacity sufficient to cause the galvanometer to deflect 411 mm., whereas by adding the same amount of antitoxin in two doses (100 + 100 per cent.) a greater deflection (485 mm.) was produced in 9 minutes (column 7). The antitoxin here used was of type (*a*), and as type (*b*) was known to be more

inhibitory towards flocculation when present in excess, we studied the effect of type (*b*) added in amounts of from 50–200 per cent. in excess of the equivalent dose. Table III shows the initial part of the reaction (with one equivalent) up to 5 minutes, which was a little more irregular than we had found in experiments with type (*a*) antitoxin. After adding the second dose of antitoxin the greatest initial effect was shown by the + 150 per cent. mixture, but after 14 minutes the + 50 per cent. mixture had the greatest opacity. In the last column (+ 200 per cent.) it is shown that the readings very slowly diminished after the 11th minute—an effect which was not observed for the other mixtures. On repeating this experiment with a greater time interval between the additions of antitoxin (100 + 200 per cent.) the results shown in Table IV and Graph II were obtained. Here the very sudden increase in opacity produced by second dose of antitoxin was followed by unmistakable reduction in the opacity.

We think that the marked reactivity of these equivalent mixtures can best be explained as follows: When toxin and antitoxin react in an equivalent mixture, the reaction product steadily aggregates and eventually flocculates. The earlier part of this aggregation stage can be followed on the photo-electric colorimeter, and we have observed that mixtures when first prepared show a negligible Tyndall effect, which steadily increases with time. The course of the reaction suggests that this aggregation increases progressively in an equivalent mixture until actual flocculation occurs. Thus, between the time of preparation and actual flocculation, the aggregated material, which may be considered as a solid phase, becomes an increasingly large proportion of the total toxin-antitoxin. This solid phase fraction, which is causing the opacity, rapidly combines with the extra antitoxin added (or part of it), and this accounts for the increased opacity of the mixture. Such combination may not lead to a condition of stable equilibrium, and there is clear evidence from Graph II that following rapid combination of the solid phase TA complex with more antitoxin, some rearrangement takes place which results in diminishing opacity. The total amount of antitoxin finally present in this mixture gives a solution of low opacity at equilibrium as can be seen from Graph III (curve A). Further evidence bearing on this point was obtained by studying the reactivity towards additional antitoxin of an equivalent mixture at varying times after preparation (Table V), and it was shown that the effect became more marked with increased time interval up to 160 minutes. By that time apparently all the toxin-antitoxin complex had aggregated into the solid phase condition, because the reactivity of the mixture kept overnight was no greater in spite of the fact that flocculation had occurred.

The reversal of the opacity has been shown to be a specific effect of antitoxin. Where the time interval between the additions was small, increasing the antitoxin to a final value of three equivalents showed chiefly the first effect, that of increased opacity, the second effect of reduced opacity not being very obvious. This can be accounted for because in the short time interval of 6 minutes only a small amount of solid phase complex was produced. With the larger time interval of 38 minutes, both the stage of increased opacity and that of diminishing opacity were marked. The ease with which an excess of antitoxin can cause the resolution of TA complex varies, for we have found

that an equivalent mixture prepared 24 hours previously, and then treated with a further two equivalents, showed only the first stage of increased opacity. Any reduction in opacity was too slow to be observed at room temperature, but it took place rapidly if the mixture was warmed to 40° C. Apparently the floccules of composition TA undergo some change with time, which makes their resolution by excess antitoxin much more difficult. This time change does not, however, appear to affect their ability to combine with additional antitoxin. Marrack (1938) comments on the fact that antigen-antibody precipitates appear to become less soluble on keeping, and states that this applies particularly to diphtheria toxin-antitoxin floccules. Ottensooser (1923) found that precipitates kept for 24 hours would not dissolve on heating to 55° C., although they did so when fresh. The nature of the changes which affect solubility in excess antitoxin are unknown.

Using the photo-electric method we have endeavoured to obtain information regarding the optical state of mixtures of toxin and antitoxin in varying proportions, under conditions which we think are equilibrium ones. The form of curve so obtained is similar to that from determination of the nitrogen content of floccules, but the optical method extends the range of observations to mixtures which are non-flocculating. On the other hand it lacks the quantitative value of nitrogen determinations on the floccules. Nevertheless Graphs III and IV show that maximal density was at approximately 1·75 and 2·5 equivalents. These figures are in very close agreement with those previously quoted for the antitoxin/toxin combining ratios of 2·6–2·8 for type (*a*), and 2·0 for type (*b*). The curves also show the remarkable manner in which any " free antitoxin " in excess of that combined with toxin caused a reduction in the opacity of the mixtures. In Graph III the curve with type (*a*) antitoxin is complicated because of interaction between the serum and a second antigen which was present in RX 3230. Graph IV shows the results for a toxoid which we believe was almost pure material, *i. e.* free from antigens other than the specific diphtheria toxoid. In both Graphs III and IV it will be seen that the curves for type (*a*) antitoxin have greater width than those for type (*b*) antitoxin, and that this difference exists chiefly on the excess antitoxin side of the equivalent point. We can offer little explanation of the fact that antitoxin of type (*a*) tends to show (1) a greater antitoxin/toxin combining ratio, (2) quicker rate of opacity production in equivalent mixtures, and (3) is less inhibitory to flocculation than type (*b*). Possibly the reduction in the size of the molecule and the removal of an inert protein fraction (Pope, 1938) leads to a general change in the properties of the antitoxin and its reaction with toxin.

Marrack (1938) has attempted an explanation of the secondary changes leading to precipitation which occur in antigen-antibody systems. He says (p. 171): " When the antibody is in excess the final aggregation will have no free antigen bonds; such an aggregate can combine with more " a " (antibody) only if it breaks up. If the combination is irreversible this should not take place." So far as the system diphtheria toxin (or toxoid) and antitoxin is concerned, our observations show that excess antitoxin can in fact break up preformed aggregates to such an extent that at equilibrium such mixtures show little optical opacity. The rate at which this break-up of preformed

aggregate takes place depends on conditions such as the age of the mixture and the temperature at which the reversal takes place.

SUMMARY.

A photo-electric method has been used to study various phases of the Ramon flocculation reaction between diphtheria toxin (or toxoid) and antitoxin.

The results obtained show that :

The rate of production of opacity is greater in a mixture containing an equivalent dose of antitoxin than in one which contains either more or less antitoxin. The rate is more affected by a decrease in the amount of antitoxin than by an increase, probably because mixtures containing two equivalents eventually develop a greater opacity than the one equivalent mixture. The rate is increased if the antitoxin is added in two fractions.

A preformed equivalent mixture gave high reactivity with more antitoxin, and under certain conditions a second stage may be observed, which is the resolution of preformed aggregates by excess antitoxin.

Mixtures of toxin and antitoxins in varying proportions at equilibrium gave opacity curves similar in shape to those for the nitrogen content of floccules of varying composition, the maxima occurring at approximately the same place.

Further studies of precipitin reactions using this method of investigation are in progress.

Our thanks are due to Mr. S. Pinfield for his assistance in the construction of the apparatus and preparation of the diagrams.

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THE EFFECT OF AGAR ON THE PRODUCTION OF STAPHYLOCOCCAL α-HÆMOLYSIN.

HENRY McILWAIN.*

From the Department of Bacterial Chemistry (Medical Research Council), Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

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AN action of agar in microbiological processes has frequently been recorded without receiving satisfactory explanation. Its stimulating action on hæmolysin production (the hæmolysin referred to throughout is staphylococcal α-hæmolysin) was first observed by Burnet (1930), and agar has been included in media used by most investigators for toxin production by *Staphylococcus*. The present investigations were carried out with the intentions of finding optimum conditions for the increase of hæmolysin by means of agar and of understanding this action, which has not previously been examined in relation to the chemical nature of agar. The effects on hæmolysin production of physical and chemical fractionation of agar and of its degradation were first determined.

The bacteriological technique was that described by Gladstone (1938), and the medium, except when otherwise stated, was the chemically defined amino-acid medium found by him to be most suited to the production of hæmolysin. The effect of agar on hæmolysin production in this medium was not so great as in the more complicated, chemically undefined media in which its action was first observed, but as the effect varied considerably in different media (see under) the chemically controlled medium was first examined. "Bacto" agar (containing approximately: water, 16–18%; ash, 2·2–2·6%; sulphur, 0·55%; nitrogen, 0·05%) was used throughout; agars not specially prepared for bacteriological work and containing more ash, nitrogen, sulphur and some water-insoluble material behaved similarly.

FRACTIONATION OF AGAR.

Physical separation.—Agar consists mainly of the calcium and magnesium salts of a mixture of polysaccharide sulphuric esters of the general formula $(RO.SO_3)_2Ca$, R representing the polysaccharide residue (Neuberg and Ohle, 1921; Percival and Somerville, 1937). The mixture is partly separable by solubility differences, and Rippel and Lehmann (1936) found fractions so separated to have different activities in stimulating bacterial growth. Fractions were separated by extracting agar with ether and alcohol, by dialysis and by water-washing, but none of these possessed greater hæmolysin stimulating

* Leverhulme Research Fellow.

properties than the original agar. The effect of a given fraction was compared with that of an equal concentration of the original agar, as shown in Fig. 1, because the effect of concentration of agar on hæmolysin production, considered below, is not straightforward. As the fractions would differ markedly in sulphur content, apparent molecular weight and setting power (Morozov, 1935), these properties could not have been of primary importance in the agar effect. It was not necessary for the agar to be present as a solid gel, for its stimulating properties were shown in concentrations which yielded only slightly viscous solutions.

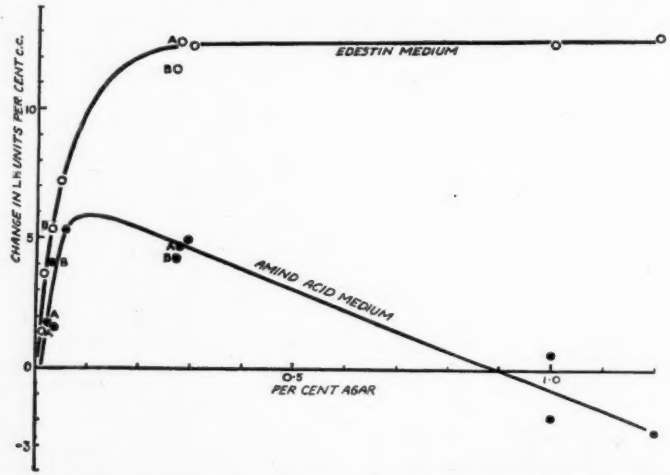

FIG. 1.—Effect of concentration of agar on hæmolysin production in amino-acid medium and in edestin medium. The value *Lh units per c.c.* is the reciprocal of the Lh dose. The figures plotted are (Lh units per c.c. in test) − (Lh units per c.c. in control). A, fractions of dialysed agar; B, fractions of washed agar.

Inorganic constituents.—The effect of the main mineral constituents was determined by fractionation of the ash. Agar (20 g.) yielded ash (0·453 g.), which was separated into SiO_2, etc. (*A*, 0·071 g.), Fe_2O_3, Al_2O_3, etc. (*B*, 0·007 g.), CaO, etc. (C, 0·130 g.), MgO, etc. (*D*, 0·073 g.). The following Lh values were obtained when these fractions and combinations of fractions were incorporated in the amino-acid medium in concentrations corresponding to 1% agar: *B*, 0·35 c.c.; *C*, 1 c.c.; *D*, 0·3 c.c.; *B* + *C* + *D*, 0·6 c.c.; *C* + *D*, 0·6 c.c.; control without added agar, 0·3 c.c. It is seen that in no case was hæmolysin production enhanced, but that all media containing the calcium fraction yielded markedly reduced quantities of hæmolysin. This effect was imitated by an equivalent concentration of calcium chloride. Inhibition by added calcium has previously been recorded (Walbum, 1922), though not by that of agar, which was probably due to the media concerned being less sensitive to added calcium than the present amino-acid medium. When the amino-acid medium was modified by the addition of hydrolysed edestin (1 per cent.; for

details see Gladstone, 1938), the inhibitory action of calcium was not observed. The effect of concentration of agar on hæmolysin formation in the amino-acid medium, shown in Fig. 1, is now explicable in terms of an opposition between this inhibitory action of calcium and a stimulating action of the remainder of the agar molecule, which together produce an optimum effect at a concentration of about 0·1 per cent. agar. On the edestin medium the unopposed stimulating action of agar increases beyond the value reached at 0·1 per cent. to a maximum, attained at about 0·3 per cent., beyond which it is not altered by considerable further increase in the amount of agar.

As agar contains two equivalents of sulphur for each of calcium or other metal, volatile acid constituents are lost on ignition. The effects on hæmolysin production of iodide, borate and arsenate, known to be present in small quantities in agar or similar materials (Kariyone and Terasaki, 1931 ; Itano and Tsugi, 1934), were therefore examined but found negative.

Calcium-free agar.—Preliminary to further work on the action of agar, it was necessary to eliminate the deleterious effect of calcium. The preparation of potassium agar, in which the calcium of normal agar is replaced by potassium by means of potassium oxalate, has been described by Fairbrother and Mastin (1923). Material prepared in this manner was found to increase hæmolysin production in the amino-acid medium without inhibition in higher concentrations (Lh values : with 1 per cent. potassium agar, 0·1 c.c. ; with 0·3 per cent. 0·25 c.c. ; control, 0·3 c.c.), but it suffered from the practical disadvantage of partial decomposition during autoclaving. A thermo-stable agar free from inorganic constituents was obtained through the sulphur-free acetyl derivative prepared by a modification of the method of Percival and Somerville (1937). The acetyl derivative was hydrolysed by alcoholic potassium hydroxide at room temperature, and the product was capable of forming a normal rigid gel, but contained negligible quantities of inorganic materials.

An agar free from sulphur and calcium was also prepared by a simpler method which took advantage of the decomposition of potassium agar on autoclaving. This was accompanied by the formation of free acid and sulphate ions, and apparently consisted initially of the hydrolysis $RO.SO_3K + H_2O \rightarrow ROH + KHSO_4$. The production of only small amounts of acid on autoclaving normal agar must be due to the greater stability of the calcium salt ; a similar phenomenon has been observed in starch (Tychowski and Masior, 1937). Reducing substances were also produced on autoclaving potassium agar which, together with the change of the material from a gel to a starch-like paste, suggested hydrolysis of the polysaccharide to have occurred. This change, similar to that caused by acid hydrolysis, was apparently due to the sulphuric acid liberated, and it was anticipated that were this neutralized as it was produced, a simple polysaccharide free from metals and sulphur would result. Using potassium carbonate, it was found possible to obtain a polysaccharide from normal agar : $(RO.SO_3)_2Ca + (COOK)_2 + K_2CO_3 \rightarrow 2ROH + Ca(COO)_2 + 2K_2SO_4 + CO_2$.

Agar (20 g.) was dissolved in hot water (600 c.c.) and potassium oxalate (8 c.c. of 10 per cent. solution) and potassium carbonate (5·2 c.c. of 10 per cent. solution) added ; the solution, opalescent by separation of calcium oxalate, was autoclaved at 130° for two hours and filtered or centrifuged clear. The product, as a solid

gel, was cut to strips and washed three times with water (3 l.) during two days and dried (yield, 12 g.; Ca, 0·01 per cent.; S, 0·05 per cent.; N, <0·003 per cent.). A 5 per cent. solution was not affected by autoclaving for three hours at 130°.

The agar polysaccharide, prepared by either of these methods, increased hæmolysin production when incorporated in the amino-acid medium and was without inhibitory action at higher concentrations (Lh with 1 per cent. polysaccharide, 0·15 c.c.; with 0·2 per cent., 0·15 c.c.; with 1 per cent. "Bacto" agar, 0·35 c.c.; with 0·2 per cent., 0·3 c.c. Control without added agar, 0·2 c.c.). The hæmolysin stimulating properties of agar are thus not associated with its sulphuric ester group, as are other of its biological properties (Bergstrom, 1930).

DEGRADATION OF AGAR.

The constituent monosaccharides of agar are not yet completely known (Neuberg and Schwietzer, 1937; Percival and Somerville, 1937). Of those which have been identified or are suspected of being present, glucose was a normal constituent of the medium, and the further addition of galactose, fructose, arabinose and xylose caused no increase in hæmolysin. An attempt was therefore made to isolate an intermediate breakdown product with hæmolysin stimulating properties. The products of hydrolysis of "Bacto" agar obtained by autoclaving at 130° for 3 hours (Takahashi and Shirahama, 1934) were separated into various fractions, all of which inhibited hæmolysin formation. Further fractionation by precipitation from aqueous solution by alcohol, by dialysis and by the formation of tannic acid complexes (decomposed for testing by extraction of the acid with alcohol) did not yield a less inhibitory material. Separate experiments showed that this result was not due to the action of the products on preformed hæmolysin.

Hydrolysis of sulphur-free agar by $N/100$ hydrochloric acid at 100° caused gradual loss of gelling power, and after a minimum of 30 minutes yielded a material which did not gel on cooling, but set to a starch-like paste (*cf.* Fairbrother and Mastin, 1923); this material, before or after the above methods of fractionation, decreased hæmolysin production. Degradation was also attempted by heating with glycerol in a manner similar to that used by Pictet and Jahn (1922) on starch. With "Bacto" agar much decomposition took place owing to the liberation of sulphuric acid, but sulphur-free agar, when heated at 160° in 5 times its weight of glycerol for a minimum time of $3\frac{1}{2}$ hours, yielded a clean material soluble in cold water to a non-gelling solution. This material, before and after separation by the previous methods, decreased hæmolysin production. The stimulating action of agar is thus lost with loss of gelling power; the inhibitory action is discussed below.

THE AGAR EFFECT AS AN ADSORPTION; DISCUSSION.

As was indicated on p. 412, the effect on hæmolysin production of incorporating a given quantity of agar in different media varied considerably. When glucose was omitted from the normal amino-acid medium, then in marked contrast to the usual stimulating action of agar, hæmolysin formation was unaffected or

decreased by its addition. This effect was given by ash-free agar, and suggested an investigation of the agar effect in relation to fermentable carbohydrate.

Glucose.—The incorporation of glucose in the amino-acid medium in amounts ranging from $M/80$ to $M/20$ caused a progressive decrease in hæmolysin (Gladstone, 1938; the effect is not due to acid-formation from the sugar). The action of agar in such media, however, was so to increase hæmolysin production that the amount formed in the presence of glucose and agar was much in excess of that in the simple amino-acid medium, the final hæmolysin increasing with increasing glucose content. Extreme instances of this effect were seen in complicated media. Hæmolysin production in broth was normally poor, but by the addition of agar was greatly enhanced, while the glycerol-broth medium of Bigger (1933), in which no hæmolysin was formed in the absence of agar, in its presence afforded a hæmolysin concentration greater than those previously described.

Thus the addition to various media of increasing quantities of fermentable substances which alone decreased hæmolysin production, in the presence also of agar caused increasing hæmolysin formation. Gladstone (1938) suggests that these higher concentrations of glucose inhibit by the accumulation of products of glycolysis during growth. McLean (1937) considers that the hæmolysin stimulating properties of agar are due to its removing from the medium by absorption compounds inhibitory to hæmolysin formation. Inhibitory substances whose effects were counteracted by agar were readily obtained from glucose by chemical means. The products obtained by heating glucose for ½ hour with $N/10$ or N NaOH or for ½–2½ hours with N HCl considerably decreased hæmolysin production. The further addition of agar restored to normal the hæmolysin. It is therefore suggested that agar counteracts the action of glucose by adsorbing deleterious metabolic products. The change in colloidal properties associated with the previous degradations of agar (the conditions of which were minimal for loss of gelling power) must alter its adsorbing powers; the lack of stimulating ability can so be understood. The additional inhibitory action is probably due to the material acting as a source of fermentable carbohydrate, for the inhibitory action of acid-hydrolysed ash-free agar was entirely removed by the further addition of whole ash-free agar to the medium.

Substitutes for agar.—Attempts to replace agar by other colloidal materials were, however, unsuccessful. Silica, starch, cellulose (filter-paper and cotton-wool), gum arabic and humic acid (prepared according to Horner, Burk and Hoover, 1935) were found almost without action on hæmolysin production, though such materials have frequently been found to simulate agar in microbiological processes (*e. g.* Rippel and Lehmann, 1936; McLean, 1937). The addition of pectin, of the soluble polysaccharide sulphuric esters prepared from cellulose by the method of Gebauer-Fulnegg (1928) and of kaolin reduced hæmolysin; the result with the latter material was probably due to its adsorbing hæmolysin from the culture (McLean, 1937). A specimen of carragheen (containing ash, 14·5 per cent.; S, 2·84 per cent.), prepared from the moss, reduced hæmolysin formation. The potassium salt behaved similarly; a sulphur-free material could not be obtained by the oxalate method, but when prepared through its acetyl derivative (*cf.* p. 413) was obtained as a white powder

(Ca, 0·05 per cent.; S, 0·14 per cent.), which like the original carragheen did not form a rigid gel. It did not increase hæmolysin production. Unfortunately no material having the same physical characters as agar is available for comparison with it.

Cellophane has recently been reported (McLean, 1937) as exceedingly effective in hæmolysin production and capable of replacing agar in this connection. These observations were made in a complex medium not chemically defined, and the present work showed that in the amino-acid medium (20 c.c.) the addition of Cellophane (2 g., British Cellophane Co. grade 300, as finely-cut strips) decreased hæmolysin production. This was accompanied by acid formation from glycerol (about 12 per cent.) present in the material, but water-washed Cellophane also decreased hæmolysin. The divergence from McLean's results is probably attributable to differences in the media employed. Thus, while agreeing with this author that agar probably acts by adsorbing deleterious materials, these are not preformed in the amino-acid medium and hence, in distinction from McLean's findings with broth media, exposure of the amino-acid medium to Cellophane before inoculation did not increase hæmolysin.

SUMMARY.

The production of staphylococcal *a*-hæmolysin in a chemically defined medium was reduced by the calcium of agar and increased by the remainder of the agar molecule.

In all media examined the hæmolysin stimulating properties of agar were not due to its sulphuric ester grouping but to the polysaccharide residue, methods of preparation of which are described. These properties were lost with loss of gelling power and not possessed by other materials examined.

Agar may act by adsorbing metabolic products deleterious to hæmolysin production though not to growth.

I am indebted to Dr. G. P. Gladstone for the considerable bacteriological work involved in these investigations, and to Dr. P. Fildes for suggestions and advice.

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THE NON-HÆMOGLOBIN CELLULAR IRON IN ANÆMIA.

C. E. JENKINS AND M. L. THOMSON.

From the Pathological Department, Salford Royal Hospital.

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In the previous (1937) paper on the distribution of iron in blood we put forward a tentative hypothesis to explain the presence of the non-hæmoglobin iron within the erythrocytes; it was suggested that the excess iron was a breakdown product derived from ageing Hb, and that the rise of the non-hæmoglobin cellular iron percentage found in most forms of anæmia was due to a compensatory retention in the circulation of cells which had passed the normal life span of an erythrocyte. The non-hæmoglobin cellular iron (N.H.C. Fe) was determined by estimating whole blood iron, subtracting the amount found in the plasma, and so arriving at the total cellular iron (T.C. Fe). The Hb was ascertained colorimetrically, and the iron present as Hb calculated by multiplying the weight of Hb per 100 c.c. of blood by 0·328 per cent., this being the proportion of iron in a molecule of Hb. The difference between the T.C. Fe and the Hb Fe is the N.H.C. Fe.

The present paper describes an attempt to test the hypothesis in greater detail. The method of determining the Fe is unchanged, but we desire to mention two additional details. The mouth of the flask should be wiped to remove any minute trace of iron rust derived from the clamp or from room dust. The second point concerns the NaOH used. Owing to the method of manufacture it is difficult to obtain NaOH entirely free from traces of iron and it is therefore wise to allow the 40 per cent. solution to stand for a week, then filter it through a piece of cotton-wool. The iron still in solution is too small to influence the measurements, and in any event the standard is prepared from the same solution as the sample.

The Newcomer method of estimating the Hb has been abandoned owing to the error caused by turbidity in samples of low Hb concentration. We now

use a 1 in 50 solution of blood saturated with CO and rendered transparent by the method of Jenkins and Don (1931).

The solution is examined with a Spekker absorptiometer and a calibrated curve using a coloured glass as a standard. Filters and water cells are always in position, and the remote possibility of a shift in the wave-length of the sensitivity curve is tested by taking the readings of five coloured glasses and solutions of various strengths in order to test the sensitivity at a number of points in the spectrum. If the readings of all the test standards remain unchanged, as they do, it may be assumed that the location and shape of the sensitivity curve is also unchanged.

In the present investigation we have endeavoured to secure untreated cases of anæmia, and to follow the change in the N.H.C. Fe percentage by periodic determinations during treatment. In this connection we desire to thank Dr. J. F. Wilkinson for his kindness in placing some of his cases at our disposal.

In order to present the data concisely each case is described separately; the Hb in grams per 100 c.c. of blood is given above the line. The N.H.C. Fe percentage is placed below it, and the interval in days between the examinations is denoted by the figure between the dots, thus:

$$\frac{\text{Hb in grammes}}{\text{N.H.C. Fe \%}} \cdot \text{days} \cdot \frac{\text{Hb in grammes}}{\text{N.H.C. Fe \%}}.$$

RECORD OF CASES.

1. Pernicious anæmia. Uneventful recovery on liver therapy:

$$\frac{4{\cdot}6}{28} \cdot 3 \cdot \frac{6{\cdot}0}{25} \cdot 7 \cdot \frac{9{\cdot}9}{21} \cdot 14 \cdot \frac{12{\cdot}3}{15} \cdot 14 \cdot \frac{15{\cdot}0}{13}.$$

2. Pernicious anæmia. Treated with liver:

$$\frac{7{\cdot}2}{18} \cdot 14 \cdot \frac{11{\cdot}5}{6} \cdot 7 \cdot \frac{12{\cdot}5}{3} \cdot 7 \cdot \frac{14{\cdot}0}{2} \cdot 7 \cdot \frac{14{\cdot}9}{2}.$$

3. Pernicious anæmia. Treated with liver:

$$\frac{8{\cdot}1}{13} \cdot 7 \cdot \frac{11{\cdot}6}{10} \cdot 7 \cdot \frac{12{\cdot}5}{10} \cdot 7 \cdot \frac{14{\cdot}2}{5} \cdot 7 \cdot \frac{14{\cdot}9}{3}.$$

4. Pernicious anæmia with Fe deficiency. At first given liver alone, then Fe in addition:

$$\frac{4{\cdot}6}{28} \cdot 1 \cdot \frac{4{\cdot}7}{26} \cdot 9 \cdot \frac{6{\cdot}0}{25} \cdot 8 \cdot \frac{9{\cdot}8}{21} \cdot 14 \cdot \frac{12{\cdot}4}{15} \cdot 21 \cdot \frac{15{\cdot}1}{12}.$$

5. Pernicious anæmia. Improved at first on liver alone, then relapsed slightly. Given Fe also and improved again :

$$\frac{6\cdot9}{35}\cdot2\cdot\frac{6\cdot9}{28}\cdot2\cdot\frac{7\cdot0}{25}\cdot10\cdot\frac{10\cdot1}{11}\cdot7\cdot\frac{9\cdot3}{21}\cdot7\cdot\frac{9\cdot4}{24}\cdot7\cdot\frac{9\cdot9}{11}\cdot7\cdot\frac{11\cdot8}{6}.$$

6. Pernicious anæmia. Liver therapy :

$$\frac{4\cdot7}{40}\cdot14\cdot\frac{8\cdot5}{31}\cdot14\cdot\frac{11\cdot0}{4}\cdot7\cdot\frac{11\cdot2}{8}.$$

7. Pernicious anæmia or aplastic. Admitted for lobar pneumonia and died. Good reticulocyte response to liver before death :

$$\frac{6\cdot2}{12}\cdot7\cdot\frac{3\cdot9}{42}\cdot7\cdot\frac{3\cdot4}{13}.$$

8. Aplastic. Intensive treatment with liver and iron ; small response at first, but later relapsed and died :

$$\frac{6\cdot3}{30}\cdot7\cdot\frac{6\cdot8}{6}\cdot14\cdot\frac{5\cdot7}{22}\cdot7\cdot\frac{4\cdot6}{30}\cdot7\cdot\frac{3\cdot7}{29}.$$

9. Aplastic. Temporary improvement on liver and iron ; was relapsing when last seen :

$$\frac{4\cdot6}{27}\cdot10\cdot\frac{4\cdot6}{16}\cdot7\cdot\frac{7\cdot5}{16}.$$

10. Scurvy. After second examination was given intensive treatment with ascorbic acid :

$$\frac{8\cdot7}{11}\cdot7\cdot\frac{9\cdot6}{13}\cdot10\cdot\frac{14\cdot6}{2}\cdot7\cdot\frac{14\cdot6}{8}.$$

11. Gastric carcinoma, with secondary microcytic anæmia. No appreciable response to iron treatment :

$$\frac{9\cdot9}{4}\cdot4\cdot\frac{9\cdot9}{6}\cdot14\cdot\frac{10\cdot8}{10}\cdot7\cdot\frac{9\cdot6}{18}\cdot14\cdot\frac{10\cdot1}{7}.$$

12. Gastric ulcer and hæmorrhage. Given ferrous iron after fourth examination :

$$\frac{3\cdot1}{38}\cdot7\cdot\frac{3\cdot2}{33}\cdot14\cdot\frac{5\cdot8}{25}\cdot3\cdot\frac{6\cdot3}{27}\cdot10\cdot\frac{7\cdot9}{20}\cdot7\cdot\frac{9\cdot9}{7}\cdot7\cdot\frac{11\cdot3}{10}.$$

13. Hæmatemesis 14 days before admission. No Fe given for 14 days, then put on ferrous Fe :

$$\frac{7\cdot2}{16}\cdot14\cdot\frac{6\cdot6}{33}\cdot7\cdot\frac{8\cdot1}{12}\cdot7\cdot\frac{9\cdot0}{3}\cdot7\cdot\frac{12\cdot2}{4}.$$

14. Large rectal hæmorrhage from hæmorrhoids 10 days before admission. Kept in bed and no anti-anæmic treatment given until spontaneous improvement ceased ; then given Fe :

$$\frac{7\cdot2}{49}\cdot7\cdot\frac{8\cdot9}{4}\cdot7\cdot\frac{10\cdot5}{4}\cdot7\cdot\frac{8\cdot4}{15}\cdot11\cdot\frac{10\cdot2}{7}\cdot21\cdot\frac{14\cdot3}{6}.$$

15. Hæmorrhoids. Large hæmorrhage on day of admission :

$$\frac{4\cdot5}{26}\cdot7\cdot\frac{2\cdot4}{50}\cdot14\cdot\frac{8\cdot5}{11}\cdot14\cdot\frac{12\cdot0}{10}.$$

16. Hæmatemesis. Had ceased bleeding on admission. Given Fe :

$$\frac{4\cdot7}{23}\cdot7\cdot\frac{6\cdot4}{10}\cdot7\cdot\frac{8\cdot4}{9}\cdot7\cdot\frac{9\cdot5}{11}.$$

17. Hæmatemesis. Ceased bleeding, but given transfusion on day of admission :

$$\frac{6\cdot9}{11}\cdot7\cdot\frac{8\cdot4}{13}\cdot7\cdot\frac{9\cdot1}{8}\cdot7\cdot\frac{10\cdot9}{11}.$$

18. Hæmatemesis 14 days before admission ; none since. Put on Maclean's diet and no Fe given :

$$\frac{9\cdot3}{6}\cdot14\cdot\frac{9\cdot2}{3}\cdot14\cdot\frac{8\cdot6}{16}.$$

19. Gastric ulcer, hæmatemesis and melæna. Severe hæmorrhage on day after second examination. Transfusion on day before fourth examination. No Fe given :

$$\frac{6\cdot9}{12}\cdot4\cdot\frac{6\cdot3}{11}\cdot7\cdot\frac{3\cdot3}{42}\cdot7\cdot\frac{6\cdot4}{8}\cdot7\cdot\frac{6\cdot5}{11}.$$

20. Gastric ulcer, hæmatemesis and melæna. Transfusion given after second examination.

$$\frac{4\cdot0}{33}\cdot4\cdot\frac{1\cdot5}{47}\cdot7\cdot\frac{3\cdot2}{25}.$$

21. Adolescent subchromic anæmia, probably dietetic. Treated with ferrous Fe :

$$\frac{6\cdot6}{25}\cdot14\cdot\frac{11\cdot0}{5}\cdot14\cdot\frac{13\cdot1}{9}\cdot7\cdot\frac{13\cdot9}{5}.$$

22. Idiopathic subchromic. Large doses of Fe at first, later reduced, then increased again :

$$\frac{8\cdot2}{16}\cdot7\cdot\frac{9\cdot4}{4}\cdot7\cdot\frac{10\cdot5}{2}\cdot7\cdot\frac{10\cdot5}{8}\cdot7\cdot\frac{10\cdot1}{10}\cdot7\cdot\frac{10\cdot5}{14}\cdot7\cdot\frac{13\cdot3}{7}.$$

23. Idiopathic subchromic. Had been improving on Fe by mouth, then remained stationary clinically, so intramuscular Fe was given with further clinical improvement :

$$\frac{9\cdot9}{3}\cdot7\cdot\frac{10\cdot0}{13}\cdot7\cdot\frac{11\cdot0}{14}\cdot7\cdot\frac{10\cdot5}{3}\cdot7\cdot\frac{11\cdot4}{2}.$$

24. Megalocytic anæmia of doubtful origin—apparently not pernicious anæmia ; test-meal normal at time of investigation. Liver therapy resulted in a slow and irregular clinical improvement. It is believed that this man died some months later of carcinoma of the stomach :

$$\frac{7\cdot9}{7}\cdot7\cdot\frac{8\cdot2}{8}\cdot7\cdot\frac{7\cdot9}{10}\cdot7\cdot\frac{10\cdot1}{10}\cdot7\cdot\frac{11\cdot3}{4}\cdot7\cdot\frac{11\cdot1}{8}\cdot7\cdot\frac{11\cdot3}{10}\cdot7\cdot\frac{12\cdot7}{7}.$$

DISCUSSION.

In this investigation 110 blood examinations were made in 24 cases. If the results be surveyed at large it will be seen that the N.H.C. Fe increases as the Hb concentration falls, and decreases as the latter rises. It was stated in the first paper that the normal N.H.C. Fe percentage was about 7 p.c. for men and a little higher for women, but it should be observed that the actual percentage is dependent upon the method of determining the Hb concentration, consequently any investigation of the N.H.C. Fe should include a specific description of the method of measuring the Hb. If this is not done it will be impossible to compare the results obtained by different workers.

The simplest method of scrutinizing the results now recorded will be to draw one or two logical conclusions from the hypothesis, assuming it to be true, and then to note how these conclusions accord with the objective facts.

If the fluctuations of the N.H.C. Fe are expressions of a compensatory mechanism, then they should in the main be independent of the cause of the anæmia. A study of the results makes it clear that this is so ; as a corollary the N.H.C. Fe is of no value as a means of differential diagnosis.

The erythrocytic population is made up of cells of varying age, and if in the course of recovery from an anæmia there is at one stage an intense hæmopoietic activity, one would expect that the new cells, having no appreciable N.H.C. Fe, would depress the percentage to a point below the normal for a brief period : a number of such instances are present (Cases 2, 6, 10, 13, 14, 22.) The result is due to the combined effects of increased removal of aged cells, in itself causing a reduction in the N.H.C. Fe percentage, and the influx of a large number of new cells. A further expectation would be that the total cellular iron might actually fall if the Hb rises sharply enough and several examples of this were encountered. The fallacy of assuming that the Hb concentration can be calculated from the total cellular iron is evident.

It is not claimed that the facts presented furnish formal experimental proof of the accuracy of the hypothesis, but we submit that all the data obtained so far can be explained rationally on that basis.

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NOTE ON THE ISOLATION OF *CL. TETANI* FROM THE INTESTINES OF NORMAL SHEEP IN CAMBRIDGESHIRE.

P. S. WATTS.

From the Institute of Animal Pathology, Cambridge.

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YOUNG (1927), in a study on the incidence of tetanus in man, found that Cambridgeshire and district had a high infection-rate. It appeared interesting, therefore, to ascertain if the same high incidence occurred in any of the common domestic animals of this district, and of these the sheep was considered the most suitable, since Kerrin (1929) had reported that 17·4 per cent. of the sheep examined by him harboured *Cl. tetani*, and Piening (1932) had also recovered the organism from 25 per cent. of sheep.

The material selected for study consisted of the separate contents of the duodenum, ileum, cæcum, colon and rectum obtained immediately after death from normal sheep slaughtered for human consumption. The methods

of isolation followed the lines described by Kerrin (1929), with the addition of animal inoculation. Every culture containing rod-shaped organisms with spherical terminal spores was injected into a mouse which had been passively immunized against *Cl. welchii*, the most common intestinal pathogen of sheep. In this way it was hoped to reveal the presence of tetanus toxin, even though, as pointed out by Tulloch (1919*a*), Ninni (1920) and Tenbroeck and Bauer (1922), its formation may be inhibited to some extent in mixed cultures.

Tubes of meat broth and *welchii*-exhausted medium were inoculated with large amounts of " neat " intestinal contents. They were then heated to 72° C. for 30 minutes, cooled rapidly and incubated anaërobically for 7 days at 37° C. A large inoculum of each culture was then transferred by a Pasteur pipette to a tube of exhausted medium (Tulloch, 1919*b*) and a peptic digest blood-agar slope (Fildes, 1925). Films were also made and examined for tetanus-like bacilli, and if these were seen 0·125 c.c. of the culture was injected into the base of the tail of a mouse. The cultures in Tulloch's medium were incubated anaërobically for 7 days and then treated in a similar manner, *i. e.* they were subcultivated on Fildes medium, examined in film preparations, and, where indicated, inoculated into mice.

All tubes of Fildes agar were examined after 24 and 48 hours' incubation for evidence of the spreading veil-like growth characteristic of *Cl. tetani*. Where this was seen a tube of meat broth was inoculated from the upper edge of the growth, but if no such growth could be detected after 48 hours' incubation, an attempt to obtain a culture from the upper part of the slope was still made in order that no growth, however delicate, should be overlooked. These meat broth tubes were incubated for 7 days at 37° C., when the cultures obtained were tested for toxicity by mouse inoculation.

In the course of this experiment 276 cultures were inoculated into mice, and of these 26 proved to be lethal. Each of the latter was then tested for the presence of tetanus toxin by injection into two groups of mice, (*a*) passively immunized against *Cl. welchii*, and (*b*) similarly protected against this organism and *Cl. tetani*. Two mice in each group were inoculated in the base of the tail with amounts of 0·25 c.c. and 0·125 c.c. respectively. Three of the cultures so tested caused death with symptoms of tetanus in the first group of mice, but had no effect on the second. They were therefore considered to be *Cl. tetani* and were purified by repeated subcultivation on Fildes agar, after which the specificity of their toxin was again checked.

The remaining 23 cultures, in which the presence of tetanus toxin had not been detected, were repeatedly subcultivated in Tulloch's medium and transferred to Fildes agar slopes. Two further strains of *Cl. tetani* were recovered by this means and their identity confirmed by animal inoculation.

SUMMARY.

Five toxigenic strains of *Cl. tetani* were obtained from separate individuals in a series of 32 sheep, one being recovered from the cæcum, two from the colon and two from the rectum. The percentage of sheep found to be

harbouring *Cl. tetani* was thus 15·6—a figure which is no higher than those recorded elsewhere. There appears, therefore, to be no particularly high carrier-rate of sheep in this area.

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THE EFFECT OF INCREASED SALT CONCENTRATION ON THE AMOUNT OF PRECIPITATE FORMED BY ANTISERA WITH SPECIFIC PRECIPITANTS.

JOHN MARRACK AND HELGA FRANZISKA HÖLLERING.

From the Hale Clinical Laboratory, London Hospital.

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THE work of Heidelberger, Kendall and Teorell (1936) on the effect of high salt concentrations on precipitin reactions has contributed valuable quantitative data on a disputed subject, besides forming a basis for a method of preparing antibodies in a pure form. However, Type III polysaccharide, which was the precipitant* in the antigen-antibody system mainly studied by them, is abnormal in its physical properties owing to the large number of COOH groups that it contains. From the theoretical point of view, further data on other systems using precipitants* which are less strongly acid may be valuable.

* Since the pneumococcal polysaccharides do not give rise to precipitating sera when used for immunization, they cannot, strictly, be called antigens. Still more, the proteins coupled with aromatic acids are not "antigens" for the Type I anti-pneumococcal sera with which they form precipitates. These substances are haptenes, but the word "haptene" is also used for the simpler substances which inhibit reactions. A term is needed for a substance which, though not the true antigen, will form a precipitate, more or less specifically, with an antiserum, and with some hesitation the word "precipitant" is used.

It has been found that specific precipitates formed with antisera to Type I and Type II pneumococci are also dissociated by strong salt solutions. But the effect of strong salt solutions on the amount of precipitate formed by antiserum to these types with the corresponding polysaccharides has not been studied quantitatively. This paper deals mainly with the effect of high salt concentrations on the amount of precipitate formed by Type II antibody and polysaccharide.

METHODS.

The horse antisera to Type I and Type II pneumococci were kindly supplied by the Wellcome Physiological Research Laboratories, and the rabbit antiserum to Type II pneumococci was given to us by Dr. Downie. The Type II polysaccharide was prepared by the method of Felton, Kaufmann and Stahl (1935); the cherry gum (weakly hydrolysed) from genuine wild cherry gum, according to the method described by Heidelberger, Avery and Goebel (1929). Antisera to proteins coupled with diazotized p-amino-benzene-arsinic acid were prepared by immunizing rabbits with horse serum-globulin antigens, containing a low percentage of arsenic; the test precipitants were made from crystallized egg albumin and contained a higher percentage of arsenic (Haurowitz, 1936–1937). Horse serum-globulin and rabbit antiglobulin serum were prepared as in Marrack and Carpenter (1938). The egg albumin compound with euxanthic acid was prepared by the method of Woolf (Woolf, Marrack and Downie, 1936).

The antisera used were mixed with the calculated amount of strong salt solution required to bring the final concentration to the desired level, and the volume made up with distilled water. Except where otherwise stated the ratio of the final volume to the volume of serum used was 6 in the experiments with horse antipneumococcal serum and Type II polysaccharide, 5 in those with rabbit serum and Type II polysaccharide, 3 in those with horse antipneumococcal serum and cherry gum, 5 in those with antiglobulin serum and 4 in those with antibenzene-arsinic acid sera. Except in certain of the latter experiments the precipitants were added to a mixture of antiserum and salt solution cooled to about 4° C. in an ice-water bath. The mixtures were kept in a refrigerator at about 0° C. overnight. The nitrogen in the precipitates, after washing twice with cold 1 per cent. NaCl solution, was estimated by a micro-Kjeldahl method. The concentration of the anion of the salt added was estimated in the supernatant fluid.

RESULTS.

Type II Anti-pneumococcal Serum and Type II Polysaccharide.

The amount of precipitate was reduced (Figs. 1 and 2) to the same degree as in the experiments of Heidelberger, Kendall and Teorell (1936). As in their experiments, the percentage reduction was greater when the antibody was in excess. However, we found magnesium and calcium chlorides reduced the amount more and sodium sulphate less than did sodium chloride solution of

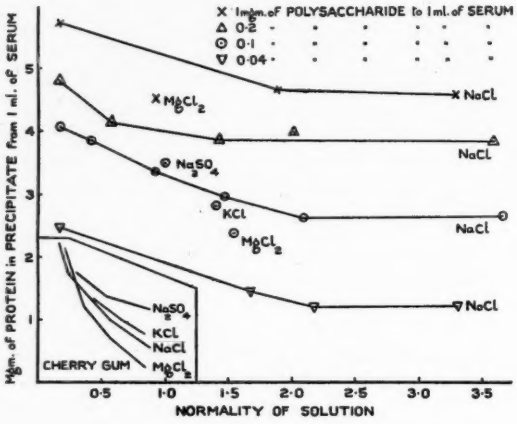

FIG. 1.—The relation between salt concentration (expressed in terms of normality) and the amount of precipitate formed by horse anti-pneumococcal serum, Type II, with Type II polysaccharide (0·1 mg. to 1 ml. of serum = slight antibody excess); and between salt concentration and the amount of precipitate formed by cherry gum with the same serum at slight antibody excess.

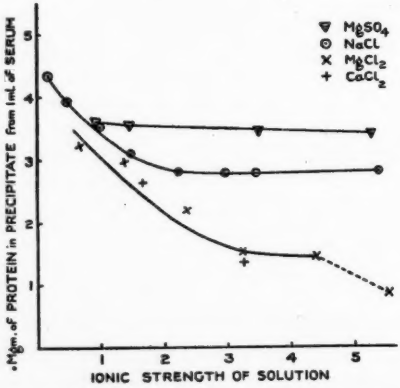

FIG. 2.—The relation between the ionic strength of salt solutions and the amount of precipitate formed by horse anti-pneumococcal serum Type II with Type II polysaccharide, 0·1 mg. to 1 ml. A different preparation of polysaccharide was used in these experiments to that used in the experiments of Fig. 1, and the amount of precipitate is uniformly slightly higher.

the same ionic strength* (Fig. 2). Heidelberger, Kendall and Teorell compared solutions of the same osmotic pressure; but their figures show that $MgCl_2$ of ionic strength approximately 0·93 did not reduce the amount of precipitate more than did a solution of NaCl of ionic strength 0·51; while a solution of Na_2SO_4 of ionic strength approximately 1·38 reduced the amount of precipitate no more than did a NaCl solution of ionic strength approximately 0·51.

An increase of the salt concentration above about 2N (ionic strength about 2 in the case of NaCl and 3 in the case of $MgCl_2$) did not produce any further reduction in the amount of precipitate, except in the highest concentration of $MgCl_2$; as noted later, a different mechanism seems to be involved in this last instance.

Glycine in concentration up to 2 M. had no appreciable effect on the amount of precipitate (Table I).

TABLE I.—*Effect of Glycine on the amount of Precipitate formed from* 1 *c.c. of Serum on the Addition of* 0·1 *mg. of Type II Polysaccharide.*

NaCl. Ionic strength.	Glycine. Approximate concentration.	Protein in precipitate. Mg. from 1 ml. of serum.
0·17	0	4·32
0·17	1·0 molar	3·9
0·17	2·0 ,,	4·2
2·2	0 ,,	2·80
2·4	2·0 ,,	2·95

In the concentrations of salt commonly used dilution has little effect on the amount of precipitate formed. The same amount of precipitate, within experimental errors, was formed when Type II polysaccharide was added to a volume of antiserum diluted, with 1 per cent. NaCl, to 1·4, 6 or 20 volumes. But in higher concentrations of NaCl and $MgCl_2$ (Table II), increase of the total volume from 2 to 6 times that of the serum used caused a considerable reduction in the amount of precipitate; further increase in the volume to 12 times that of the serum caused little or no further reduction. This dilution effect might be ascribed to an opposition effect of other diffusible ions in the serum, the effect of which might disappear when the serum was further diluted. However, this was excluded by the experiments in which serum, dialysed against 1 per cent. NaCl solution, was used. Nor was this dilution effect due to the high concentration of non-specific protein in the smaller volume, for the effect was observed when antibody concentrate, prepared by the Felton method, was used. This concentrate contained 0·87 per cent. of non-specific protein, while the untreated serum contained 5·6 per cent.

In the experiments of Heildelberger, Kendall and Teorell the effect of high salt concentration was greater with rabbit antibody than with horse antibody.

* The ionic strength of a solution is equal to $\frac{1}{2} \Sigma n_a v_a^2$, where n_a is the concentration (reckoned as number of gram ions per 1000 grams of water) and v_a the valency of ions of species "a". Thus the ionic strength of a solution which contains 1 g. molecule of salt per 1000 g. of water is 1 when the salt is NaCl, 3 when the salt is $MgCl_2$ or Na_2SO_4 and 4 when the salt is $MgSO_4$.

TABLE II.—*The Effect of Dilution in the Presence of High Salt Concentrations.*

Antibody used.	Salt.	Ionic strength.	Final volume (ml.).	Protein in precipitate. Mg. from 1 ml. of antibody.
Serum	NaCl	0·92	2	4·18
		0·98	6	3·62
		1·13	6*	3·3
		1·13	12	3·31
		1·35	2	3·85
		1·35	6*	3·1
		1·47	6	3·10
		1·47	12	3·05
		2·0	2	3·28
		2·0	6*	2·85
		2·0	12	2·90
		2·8	2	3·50
		2·8	6*	2·8
		3·2	6*	2·8
		..	12	2·76
Serum	$MgCl_2$	2·55	2	2·90
		2·36	6	2·20
		2·36	12	2·18
Felton antibody	NaCl	0·17	6	5·7
		2·8	2	3·50
		2·8	12	3·0
Dialysed serum	NaCl	0·17	6	3·99
		1·48	2	2·77
		1·46	12	2·60
		1·75	2	2·85
		1·75	12	2·48
		2·2	2	2·70
		2·1	12	2·50

* Calculated from the curve, Fig. 2.

In the two experiments which we made with rabbit serum, with antibody in slight excess (Table III), the effect was less than with horse serum.

The supernatant fluids removed from the precipitates formed in the horse-serum experiments with NaCl (ionic strength 4·9) and $MgCl_2$ (ionic strength 3·22, 4·4 and 5·55) were dialysed against 1 per cent. NaCl solution in the cold. No precipitate formed in these fluids on dialysis except in that from the experiment with $MgCl_2$ of ionic strength 5·55.

Anti-pneumococcal Serum and Cherry Gum.

Appreciable reduction was effected by concentrations of salt that had little effect on the amount of precipitate formed with Type II polysaccharide. The concentrations shown in the figure (Fig. 1) are calculated in normality. But NaCl solutions had approximately the same effect as $MgCl_2$ solutions of the

same *ionic strength*, and a much greater effect than Na_2SO_4 solutions of the same ionic strength. KCl had less effect than NaCl.

However, the salt effect in the cherry gum experiments was not strictly comparable with that in the experiments with pneumococcal polysaccharides; for precipitates formed in the supernatant fluids produced in the experiments with 0·59 and 0·85 N. $MgCl_2$, when these were dialysed against 1 per cent. NaCl solution (Table III, "on dialysis", 4th column).

TABLE III.— *Effect of Salt Concentration on the amount of Precipitate formed by Immune Sera with Various Precipitants.*

Serum.	Precipitant.	Salt.	Ionic strength.	Protein precipitated. Mg. per 1 ml. of serum.
Rabbit, anti-pneumococcal Type II	Type II polysaccharide	NaCl	0·18	5·31.
,,	,,	,,	1·25	4·78.
,,	,,	$MgCl_2$	1·4	4·63.
Horse, anti-pneumococcal Type II	Cherry gum	,,	0·89	0·695 0·73 on dialysis.
,,	,,	,,	1·27	0·23. 1·13 on dialysis.
Horse, anti-pneumococcal Type I	Metanilic acid protein 4·65 mg.	NaCl	0·10	11·15. Total precipitant. 3·62. Precipitant.
,,	,,	,,	0·17	7·25. Total precipitate. 2·55. Precipitant.
,,	Metanilic acid protein 9·37 mg.	,,	0·10	14·6. Total precipitate. 6·0. Precipitant.
,,	,,	,,	0·17	8·6. Total precipitate. 4·04. Precipitant.
Rabbit, anti-horse serum globulin (slight antibody excess)	Horse-serum globulin	,,	0·17	3·16.
	,,	,,	1·4	2·66.
	,,	,,	2·9	3·08.
	,,	,,	3·0	2·8.
	,,	$MgCl_2$	0·37	2·74.
	,,	,,	0·81	3·26. Total volume 2·2 ml.
	,,	,,	0·92–4·4	Cloudy, no precipitate.
Rabbit, anti-azo-benzene-arsinic acid protein (slight antibody excess)	Egg-albumin-azo-benzene-arsinic acid	NaCl	0·17	7·65. Total precipitate. 0·98. Precipitant.
		,,	0·38	7·5. Total precipitate. 0·98. Precipitant.
	,,	,,	0·80	6·22. Total precipitate. 0·98. Precipitant.
	,,	,,	1·8	5·76. Total precipitate. 0·98. Precipitant.

Increased sodium chloride concentration reduced the amount of precipitate formed by Type II antiserum with protein combined to euxanthic acid to as great a degree as that formed with cherry gum.

Horse-Serum Globulin and Rabbit Antiserum.

The amount of precipitate was little effected by high concentrations of NaCl (Table III). But in $MgCl_2$ solutions (0·92 to 4·4) milky suspensions formed; solid precipitated slowly from the first two on keeping for several days. When the final volume was only 2·2 that of the serum used, the amount of precipitate was not reduced by $MgCl_2$, ionic strength 0·81.

Egg-albumin-azo-benzene-arsinic Acid and Rabbit Antiserum.

In experiments at 0–5° C. the amount of precipitate was reduced, when the salt concentration was increased (Table III), to much the same degree as the Type II polysaccharide-antibody precipitate. The amount of precipitant (estimate colorimetrically) in the precipitate was not reduced. With a weaker serum the reduction in the amount of precipitate was greater but the results less regular. At room temperature the results were inconstant. On one occasion no precipitate formed, on standing overnight, in tubes containing 0·46 N. and 0·7 N. NaCl. On keeping these tubes for 24 hours in the refrigerator very little precipitate formed, and a little more than the contents were dialysed against 1 per cent. NaCl solution. When these experiments were repeated, precipitates formed as in the experiments in Table III.

DISCUSSION.

The amounts of precipitate formed by horse anti-pneumococcal sera Type II with Type II polysaccharide, and by proteins coupled with diazo-benzene-arsinic acid and the corresponding antibody were reduced, with increasing salt concentration, to the same degree as the precipitate formed by Type III pneumococcal antisera and Type III polysaccharide. In our experiments the precipitates formed with rabbit anti-pneumococcal serum were less reduced by high salt concentration. But free antibody can be recovered from the specific precipitates from rabbit antisera to Type I pneumococci, by extraction with concentrated salt solutions (Heidelberger and Kabat, 1938); qualitatively, therefore, the effect of high salt concentration on the specific precipitates from rabbit antisera is not confined to Type III antisera. The antibodies in these rabbit sera have not the remarkable physical properties of horse anti-pneumococcal antibodies. The salt effect, therefore, does not depend on the physical properties of the antibodies.

The uronic acids are apparently essential parts of the determinant groups of the pneumococcal polysaccharides; benzene-arsinic acid is the determinant group of the compounds of proteins with diazo-benzene-arsinic acid and metanilic acid of the proteins coupled with m-diazo-benzene-sulphonic acid. The salt effect has, therefore, been demonstrated in reactions with precipitants,

the determinant groups of which are acid. It would be interesting to examine the effect of high salt concentrations of the amount on precipitate in other systems in which the precipitants were other neutral polysaccharides or synthetic protein compounds with acid determinant groups.

As Heidelberger, Kendall and Teorell point out, the dialysis experiments show that the diminution of the amount of protein precipitated is not due to increased solubility of the precipitate. In the experiments with antisera to p-amino-benzene-arsinic acid derivatives, the same conclusion may be drawn from the fact that the amount of precipitant in the precipitate was not reduced. The cause appears to be a shift in the proportions in which the precipitant and antibody combine. Heidelberger, Kendall and Teorell suggest that this may be due to a competition of the cation with antibody for combination with the polysaccharide anion, or a driving back of the ionization of the soluble antibody-sodium chloride complex. This amounts to a reduction of the activity of the combining groups of the precipitant or of the antibody. The differences found between Mg, Ca and Na salts and between sulphates and chlorides are also seen in the effects of salts on the activity of proteins (Green, 1932) and cystine (Cohn *et al.*, 1938). In the equation of Heidelberger and Kendall (1935),

$$y = 2\,Rs + \frac{R^2}{A}s^2$$

(where y is the amount of antibody nitrogen precipitated, s the amount of polysaccharide in the mixture, A the amount of antibody nitrogen), we can substitute $f_n s$ for s and $F_n A$ for A (where f_n and F_n are the activity coefficients of the polysaccharide and of the antibody respectively in salt of ionic strength n); f_n and F_n can then be calculated from the equations given by Heidelberger, Kendall and Teorell. The values so calculated would indicate the F_n falls slowly with rising values of n; but that f_n is more affected by the ionic strength. The values found for f_n could fit the Debye-Hückel equation, with low values of the constants—the ionic charge of the determinant group, the "mean ionic diameter" and the "salting out" coefficient. The values found for f_n should be independent of the nature of the antibody (if the same determinant groups are involved). However, the value of f_n, when $n = 0{\cdot}93$ approximately, calculated from the experiments with rabbit antibody, is considerably lower than that calculated from the experiments with horse antibody. A correction for the reduction of activity of the precipitant and antibody cannot therefore be made in this simple way. It would also be expected that if the activity coefficient was affected by salt concentrations according to the Debye-Hückel theory, it would also be altered in high concentrations of glycine, owing to the change in the dielectric constant (*cf.* Cohn *et al.*, 1938); we found no evidence of this.

It is interesting to compare this salt effect with the inhibitory action of simple haptenes, which are supposed to compete with the determinant groups of the precipitant for combination with the antibody. In unpublished experiments, made in this laboratory, Dr. B. Woolf has measured the amount of precipitant and antibody in the precipitate formed in the presence of varying amounts of simple haptene. Using antiserum to horse-serum globulin coupled

to diazotized p-amino-benzene-arsinic acid, with p-amino-benzene-arsinic acid as the haptene and the egg albumin compounds as precipitant, he found that both precipitant and antibody were reduced in the precipitate formed in the presence of haptene, but that the ratio of precipitant to antibody in the precipitate was increased.

Heidelberger, Kendall and Teorell also suggest that the Coulomb forces due to the heaping-up of carboxyl groups in the Type III polysaccharide molecule result in a spatial configuration which is altered when these charges are reduced by high salt concentrations, with a consequent diminution of reactivity with correspondingly spatially oriented groupings in the antibody molecule. If this were the explanation it would be expected high salt concentrations would have less effect on the amount of precipitate formed by Type II polysaccharide with antibody, since Type II polysaccharide contains fewer carboxyl groups than Type III.

In the experiments in which natural proteins were used as antigens, Heidelberger, Kendall and Teorell obtained milky suspensions with high salt concentrations when antigen was in excess ; we also obtained such suspensions, with slight antibody excess, in the higher concentrations of $MgCl_2$. It appears that in these systems the high concentration of salt does not affect the combination of antibody and antigen, but inhibits a later stage of the process ; this suggests that the later stages of the aggregation of the antigen-antibody complex does not depend on the specific combination between antigen and antibody, as maintained by Marrack (1934) and Heidelberger and Kendall (1935).

Downs and Gottlieb (1933) found that 1 N. NaCl completely inhibited precipitate formation by egg-albumin and the corresponding antiserum. This was probably due to failure of the later stages of aggregation, as in the experiments of Heidelberger, Kendall and Teorell with antigen excess, and those of ourselves with higher concentrations of $MgCl_2$. While the inhibition of precipitation of dilute p-amino-benzene-arsinic acid antiserum and precipitant observed by Haurowitz (1936–1937), when the NaCl concentration was raised to 4 per cent., is comparable to the inconstant results obtained by us in the reaction between this antiserum and precipitant at room temperature.

The reduction of the amount of precipitate formed in the cross reactions (Type II antiserum with cherry gum and euxanthic protein, and Type I antiserum with sulphanilic acid protein, Table III) is partly due to increased solubility of the precipitates in high salt concentrations. The effect of salt concentration is so great that care must be taken to keep this constant in any quantitative experiments on cross reactions.

Our thanks are due to the Wellcome Research Laboratories for a supply of horse anti-pneumococcal sera and to Dr. Downie for rabbit sera.

CONCLUSIONS.

1. The amounts of precipitate formed by Type II pneumococcal antiserum with Type II polysaccharide and by antiserum to p-amino-benzene-arsinic acid proteins with egg-albumin-azo-benzene-arsinic acid are reduced in high salt concentrations. The amount of this reduction is as great as that found by Heidelberger, Kendall and Teorell (1936) with Type III antibody and

polysaccharide. The reduction in the amount of precipitate is not due to increased solubility of the precipitate in high concentrations of salt.

2. Magnesium and calcium chloride are more effective and magnesium and sodium sulphate less effective than sodium chloride in the same ionic strength.

3. The salt effect is greater when antibody is in excess than at equilibrium or when precipitant is in excess.

4. High salt concentrations of sodium chloride do not produce similar reduction of the amount of precipitate formed by horse-serum globulin with the homologous rabbit antiserum.

5. The amount of precipitate formed in cross reactions between Type II pneumococcal antiserum and cherry gum or protein euxanthic acid compound, and between Type I serum and protein-metanilic acid compound, are reduced by relatively slight increase of salt concentration. This reduction is partly due to an increased solubility of the precipitate.

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THE INFLUENCE OF INTRAVENOUS INJECTIONS OF 3 : 4-BENZPYRENE COLLOID UPON THE GROWTH OF TRANSPLANTED MOUSE SQUAMOUS CARCINOMA 2146.

P. R. PEACOCK AND STEPHAN BECK.*

From the Research Department of the Glasgow Royal Cancer Hospital.

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SINCE Haddow's discovery of the inhibitory effect of carcinogenic hydrocarbons upon tumour growth many other investigators have confirmed his observation. In such experiments, intraperitoneal, subcutaneous or intratumoral injections were employed, so that, while the hydrocarbons inhibited the tumour growth, they formed deposits which, in all probability, would have led to further new growth. It was thought that the risk of carcinogenesis, due to 3 : 4-benzpyrene, might be overcome by injecting it intravenously as a colloid.

PREVIOUS WORK.

Haddow (1935) described the inhibitory effect of 1 : 2 : 5 : 6-dibenzanthracene, 3 : 4-benzpyrene and other hydrocarbons upon the growth of Jensen rat sarcoma, Walker carcinoma and a rat sarcoma induced by 1 : 2 : 5 : 6-dibenzanthracene. Such an effect was not shown by non-carcinogenic hydrocarbons, amongst them anthracene. In continuing these experiments, Haddow and Robinson (1937) found that all definitely carcinogenic hydrocarbons had an inhibitory effect upon the Jensen and Walker tumours, and that feebly carcinogenic hydrocarbons also showed an inhibiting power. In rats, treated with carcinogenic hydrocarbons, the growth of the body was remarkably retarded. Morelli and Dansi (1936) confirmed that 1 : 2 : 5 : 6-dibenzanthracene and 3 : 4-benzpyrene had a strongly inhibiting effect upon grafted tumours in rats. The inhibition was greatest if the injections were made simultaneously with the graft. These results were confirmed by Lees (1937) as to the effect of intraperitoneal injections of 1 : 2 : 5 : 6-dibenzanthracene upon the growth of the Jensen sarcoma. An inhibitory effect of 1 : 2 : 5 : 6-dibenzanthracene upon grafted rat sarcoma is reported by Pollia (1937) to have occurred in every case in his experience. Alapy (1938) reported attempts at prophylaxis with very small doses of benzpyrene, dibenzanthracene, and methylcholanthrene (1·5γ to 3γ for mice). His best results were obtained when he injected the hydrocarbons thirty to forty days before tumour inoculation. The inhibition, however, was only slight in mice and not appreciable in rats. The action of carcinogenic hydrocarbons on mice bearing spontaneous mammary tumours was studied by Pybus and Miller (1937). After repeated intraperitoneal injections of 1 : 2 : 5 : 6-dibenzanthracene in water, gum saline, or olive oil, giving a total dosage of 10 mg., eight partial or complete regressions occurred among sixty mice. The effect in most cases in their experiments was only temporary, and regression of one tumour might be

* Working under a grant from the International Cancer Research Foundation.

contemporary with the growth of another mammary tumour in the same animal. The inhibitory effect of 3 : 4-benzpyrene in human cases was examined by Bauer (1937), who injected 0·5 to 1·0 c.c. of a 0·5 per cent. solution of this hydrocarbon in ether into the tumours, and sprayed the solution on the surface of superficial growths. Seven out of twenty-three cases were reported to have remained healed for over two years. A contrary effect of subcutaneous injections of 1 : 2 : 5 : 6-dibenzanthracene was recorded by Appel, Strauss, Kolischer and Nacheles (1938), who found that the injections stimulated the growth of the Brown-Pearce rabbit carcinoma, and caused earlier metastasis and secondary tumours in organs otherwise seldom involved.

METHODS.

Mouse squamous carcinoma 2146* was used as a transplantable tumour. Every week eighteen mice were injected subcutaneously with 0·25 c.c. of a 1/10 suspension of this tumour in saline. Within the next few days (mostly on the following day) daily intravenous injections were commenced, six mice receiving 0·03 per cent. benzpyrene colloid, six 0·06 per cent. anthracene colloid, and six were kept as controls. Altogether, 350 mice were used. In addition, twenty-eight mice without tumours were injected with 0·03 per cent. 3 : 4-benzpyrene colloid, kept under observation until death occurred, and then examined post-mortem. The colloids of the hydrocarbons were prepared by precipitation of the hydrocarbons from solution in acetone, which was then removed by dialysis. In our more recent experiments 0·3 per cent. gelatine was used. The repeated injections were continued as long as possible, but thrombosis of the tail veins occurred sooner or later, and determined the number and frequency of the injections.

RESULTS.

Those mice only which received at least eight injections of either hydrocarbon are recorded in the figures. Fig. 1 shows the results of 3 : 4-benzpyrene colloid injections ; Fig. 2, those of anthracene colloid, used as a control. In both figures the days are given on the horizontal line below the chart. Each line represents the lifetime of a mouse. The black area represents the growth of the tumour, the maximum diameter of which is indicated by the height of the curve. The vertical lines represent the injections of colloid on the respective days, and the length of these lines shows the amount of the hydrocarbons injected. It will be seen that complete or partial regressions followed 3 : 4-benzpyrene colloid injections in six of thirteen mice so treated. Data for these regressed tumours are given in Table I. Mice bearing progressively growing tumours were not allowed to die from complications, but were killed as soon as the tumours showed signs of ulceration.

Mouse carcinoma 2146 injected subcutaneously gave, in our experiments, about 70 per cent. of progressively growing tumours. Two out of 120 controls in this experiment resulted in small palpable nodules which disappeared within about 10 days. It is not certain that these were true regressing tumours, and they were quite unlike those tumours classed as regressing, following

* Carcinoma 2146 of the Imperial Cancer Research Fund. Animals bearing this strain were kindly supplied on two occasions by Drs. Gye and Cramer.

benzpyrene colloid treatment. As far as the results with benzpyrene colloid are concerned, only the regressions of well-defined tumours are recorded; regression began about 14 days after tumour inoculations and took variable lengths of time to become complete, as seen in Fig. 1.

A tumour regressed in Mouse No. 41, which received five injections, or a total amount of 0·54 mg. anthracene. This tumour was 2 mm. in diameter, and regressed after 10 days. This regression occurred at a very early stage, similar to that of the two controls.

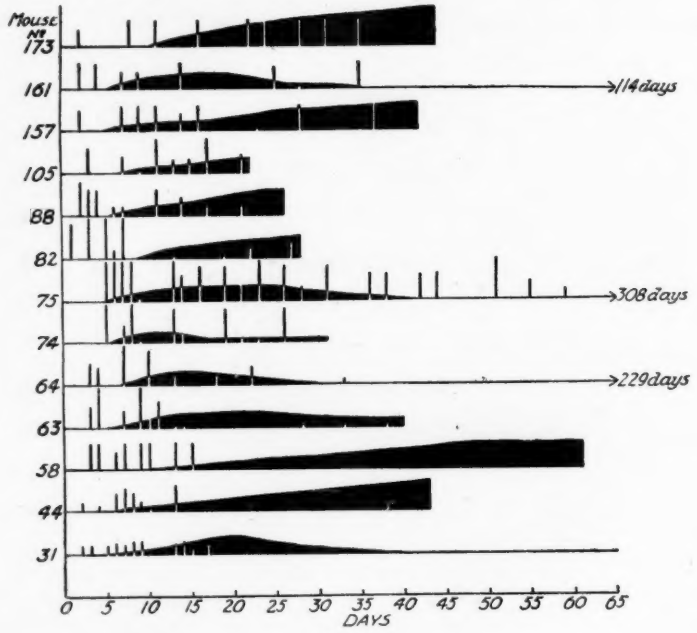

FIG. 1.

The mice, Nos. 31 and 64, were unsuccessfully re-injected with tumour suspension 8 days after the complete regression of the tumour; control mice, injected with the same amount of this suspension, developed tumours.

Mouse No. 62, which was not inoculated with tumour but which received 11 injections of 3 : 4-benzpyrene colloid, a total amount of 2·1 mg. benzpyrene, died 354 days after the first injection, and, at post-mortem examination, showed three adenomata of the lung. Mice painted with tar or treated by intraperitoneal injections of benzpyrene showed similar adenomata. So far no untreated mouse in our laboratory has produced such an adenoma, though lungs were examined in more than 400 control mice. Spontaneous adenoma of the lung is, of course, known to occur in mice, but the multiplicity of the tumours in this case suggests that they were related to the benzpyrene treatment.

In mouse No. 225, during intravenous injection, a part of the 0·6 c.c. of

benzpyrene colloid was seen to have leaked into the subcutaneous tissue near the root of the tail. After 258 days a sarcoma was observed at this site.

Toxicity.

The toxicity of 3 : 4-benzpyrene colloid appears to differ in the several species of animal tested. In the only human being tested by one of us (P. R. P) thirty-one intravenous injections, each containing 6 mg. 3 : 4-benzpyrene in colloidal solution, within 39 days, were tolerated without subjective or objective signs of toxicity. This case was one of rapidly-growing and fatal sarcoma of the nose, and was not apparently influenced by the benzpyrene colloid injections.

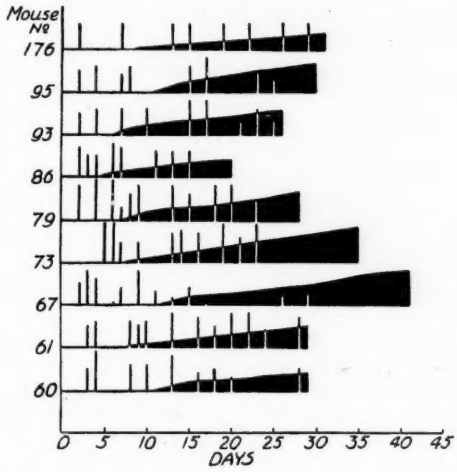

FIG. 2.

There were no toxic symptoms, either in mice receiving daily injections of 0·3 mg. benzpyrene, or in rabbits or fowls receiving 0·5 mg. benzpyrene.

Chalmers, working in this Department, found hæmoglobinuria in rats after injections of 0·12 to 0·6 mg. benzpyrene. One of us (P. R. P.) found, in experiments on goats, that 90 mg. benzpyrene in colloidal solution intravenously injected was followed by death of the animal, with acute hæmaturia and hæmorrhage from the lung, one hour after injection. Toxic symptoms were previously observed in two goats, following the intravenous injections of 10 c.c. and 30 c.c. of 0·3 per cent. benzpyrene colloid respectively.

DISCUSSION.

There is evidence that the inhibitory effect of intravenous injections of 3 : 4-benzpyrene colloid does not depend so much on the total amount given as on the frequency of the injections shortly after tumour transplantation. This observation may be explained by the fact that even large doses of intravenously injected benzpyrene are quickly eliminated (Peacock, 1936, Chalmers and Peacock, 1936). It is to be noted that the inhibitory effect was obtained

with smaller total amounts of 3 : 4-benzpyrene, injected intravenously, than was the case with intraperitoneal or subcutaneous injections. While the absorption from intraperitoneal or subcutaneous injections is rather slow, especially when fatty solvents are used, the intravenous injection throws the hydrocarbon into solution in the fatty tissues of the mouse, and probably into the lipoids of the tumour, during several hours after injection, while elimination by the liver is taking place.

TABLE I.

Mouse number.	Days of injection.	0·03 per cent. benzpyrene colloid (c.c.).	Benzpyrene (mg.).
31	2	0·2	0·06
	3	0·2	0·06
	5	0·2	0·06
	6	0·25	0·075
	7	0·2	0·06
	8	0·25	0·075
	9	0·3	0·09
	13	0·2	0·06
	14	0·3	0·09
	15	0·1	0·03
	17	0·2	0·06
		Total	0·72
63	4	0·5	0·05
	5	1·0	0·3
	8	0·4	0·12
	10	1·0	0·3
	11	0·2	0·06
	12	0·6	0·18
	14	Trace	0·01
	15	,,	0·01
	29	0·1	0·03
	34	0·1	0·03
	39	0·1	0·03
		Total	1·12
64	3	0·5	0·15
	4	0·4	0·12
	7	1·0	0·3
	10	0·8	0·24
	13	0·2	0·06
	18	0·2	0·06
	22	0·4	0·12
	33	0·1	0·03
		Total	1·08

TABLE I (*continued*).

Mouse number.	Days of injection.	0·03 per cent. benzpyrene colloid (c.c.).	Benzpyrene (mg.).
74	5	1·0	0·3
	7	0·4	0·12
	8	1·0	0·3
	9	Trace	0·01
	13	0·8	0·24
	14	0·2	0·06
	19	0·8	0·24
	21	0·1	0·03
	23	Trace	0·01
	26	0·8	0·24
		Total	1·55
75	5	1·0	0·3
	6	1·0	0·3
	7	1·0	0·3
	8	1·0	0·3
	9	1·0	0·3
	13	1·0	0·3
	14	0·6	0·18
	16	0·8	0·24
	19	0·8	0·24
	21	0·4	0·12
	23	1·0	0·3
	26	0·8	0·24
	28	0·3	0·09
	31	0·8	0·24
	36	0·6	0·18
	38	0·6	0·18
	42	0·6	0·18
		Total	3·99
161	2	0·6	0·18
	4	0·6	0·18
	7	0·4	0·12
	9	0·4	0·12
	14	0·6	0·18
	25	0·5	0·15
	28	Trace	0·01
	35	0·6	0·18
		Total	1·12

Histological confirmation of the regressing tumours was not possible from the nature of the experiment ; but, in No. 63, death occurred from intercurrent disease while regression of the tumour was incomplete, and the tumour was histologically confirmed. No. 74, at death, had a small necrotic tumour, about 2 mm. in diameter, which was not examined histologically.

The fact that Nos. 31 and 64 were unsuccessfully re-inoculated, as described above, indicates that immunity to carcinoma 2146 had developed, and thus confirms the nature of the treated tumours.

While the number of successful treatments is small, it is thought to be significant in view of the control experiments, but it is doubtful if a much higher proportion of successful results could be obtained in this type of experiment, owing to the difficulty of maintaining frequent intravenous injections in mice.

Moreover, the attempts to eliminate the risk of carcinogenesis from benzpyrene injections by employing the intravenous route cannot be considered successful. We have already mentioned three lung adenomata occurring in one mouse 354 days after the first injection. Fluoroscopic examination of the lungs of mice shortly after intravenous injections of benzpyrene colloid revealed fluorescent areas corresponding with deposits of benzpyrene in the capillaries. These may persist and may be responsible for the induction of such multiple adenomata.

SUMMARY.

1. There is evidence that intravenous injections of 3 : 4-benzpyrene colloid can inhibit transplanted mouse squamous carcinoma 2146, success depending more on the frequency than on the amount of the injections ; in six out of thirteen mice that received eight or more injections of colloid, four tumours regressed completely and two partially.

2. The toxicity of intravenously injected benzpyrene colloid differs in the various species of animals tested, and was low in mice, rabbits and fowls (and in one human being) and high in rats and goats.

3. Intravenous injection of benzpyrene colloid is not harmless ; three lung adenomata observed in a mouse 354 days after the first injection were probably attributable to the treatment. Deposits of benzpyrene can be demonstrated in the lungs of mice shortly after intravenous injection of benzpyrene colloid.

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